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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C12N 5/00

A2

(11) International Publication Number: WO 00/15764

(43) International Publication Date: 23 March 2000 (23.03.00)

(21) International Application Number: PCT/GB99/03031

(22) International Filing Date: 13 September 1999 (13.09.99)

(30) Priority Data:
9819912.8 11 September 1998 (11.09.98) GB

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(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

Without international search report and to be republished upon receipt of that report.

(54) Title: PROPAGATION AND/OR DERIVATION OF EMBRYONIC STEM CELLS

(57) Abstract

Embryonic stem (ES) cells are cultured in the presence of a compound which selectively inhibits propagation or survival of cells other than ES cells. The ES cells have not been genetically altered. Instead, the compound inhibits a signalling pathway which is essential for propagation of differentiated cells but is not essential for propagation of ES cells – hence ES cells are selectively maintained in the culture.

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PROPAGATION AND/OR DERIVATION OF EMBRYONIC STEM CELLS

The present invention relates to propagation and/or derivation of embryonic stem (ES) cells and to compositions therefor.

Stem cell self-renewal underpins growth and diversification during development of the mammalian embryo and tissue repair and homeostasis in the adult. However, studies on stem cell biology have been hampered by the absence of normal, non-transformed stem cells that can be propagated in vitro. An exception to this are mouse ES cells, which can be cultured indefinitely as pluripotential stem cells when the medium is supplemented with a ligand that activates the cytokine receptor gp130. These stem cells are present only transiently in the early embryo. However, they are intrinsically tumorigenic and give rise to stem cell tumours, teratocarcinomas, when early embryos are grafted to ectopic sites. Furthermore, when the epiblast of a mouse blastocyst is explanted in culture, immortal embryonic stem (ES) cell lines can be derived.

Propagation of ES cells is dependent on the presence of the cytokine LIF, which promotes the proliferation of undifferentiated stem cells through the activation of a heteromeric complex containing two related cytokine receptors, gp130 and the low affinity LIF receptor, LIF-R.

Signal transduction via gp130 depends upon the activation of JAK kinases, a class of non-receptor tyrosine kinases that associate with the membrane proximal box1/box2 region of cytokine receptors. Upon activation, JAKs phosphorylate tyrosines in the intracellular domain of gp130 creating binding sites for proteins containing Src-homology-2 (SH2) domains. These proteins can in turn be phosphorylated, resulting in the activation of a variety of signalling molecules, including STATs (signal transducer and activator of transcription) 1 and 3, the tyrosine phosphatase SHP-2, the

mitogen activated protein kinases ERK1 and ERK2, insulin receptor substrate-1(IRS-1), Grb2 associated docking protein (Gab1) and phosphatidylinositol (PI)-3 kinase and the non-receptor tyrosine kinases hck and btk. Amongst these, both the STAT and MAPK signalling pathways have been demonstrated to play essential roles in mediating the biological responses to ligands that activate gp130 in various cell types.

STATs are a family of latent transcription factors that upon recruitment to a receptor become phosphorylated, dimerise and then translocate to the nucleus where they regulate transcription of target genes. We have recently shown that activation of STAT3 is required for maintaining the pluripotent phenotype of ES cells. Chimeric gp130 receptors unable to engage STAT3 were incapable of signalling self-renewal, whilst over-expression of a STAT3 interfering mutant caused ES cells to differentiate. However, in the absence of constitutively active forms of STAT3, we have been unable to determine whether this regulator alone is sufficient or if other signals are also required for self-renewal.

gp130 can also associate with the protein tyrosine phosphatase. This widely expressed enzyme has also been implicated in signal transduction from receptor tyrosine kinases (RTKs) and is regarded as a positive effector of the ERK signalling cascade. Although the biologically relevant substrates for SHP-2 phosphatase have not been unequivocally identified, it is significant that over-expression of catalytically inactive SHP-2 mutants can suppress receptor mediated activation of the ERK pathway. Recruitment to the gp130 receptor complex also results in tyrosine phosphorylation of SHP-2. These phosphotyrosines can serve as binding sites for the adaptor protein Grb2, which potentially couples the receptor, through interactions with SOS and Ras, to the ERK pathway. Stimulation of ERK1 and ERK2 has been demonstrated to play a role in mediating mitogenic responses of cells to growth factors, though the precise nature of this role is yet to be elucidated.

It is known to maintain cultures of ES cells in the presence of certain factors that specifically promote proliferation of ES cells, and LIF is one such factor. This does not, however, absolutely prevent differentiation; there is a continuing loss of ES cells from these cultures which accordingly in time become overgrown with differentiated cells. Thus, it remains a problem to reduce even the small rate of differentiation of ES cells when propagated in such known factors.

A further problem in this art is that the range of ES cells that can be propagated in culture is limited to just a few types, mainly mouse ES cells, despite efforts to derive ES cells from other species.

It is also known to introduce into ES cells a selectable marker that is differentially expressed in (i) ES cells and (ii) cells other than ES cells. Selection can then be used to eliminate those cells that have differentiated. But, this requires genetic alteration of the ES cells.

An object of the invention is to provide an alternative method of obtaining and/or culturing ES cells. Another object of the invention is to reduce the rate of differentiation of ES cells in known cultures. A further object is to provide culture medium components for maintenance or derivation of a culture of ES cells.

The present invention is based upon the discovery of a class of compounds that selectively promote self renewal of ES cells and/or inhibit propagation or survival of cells other than ES cells, ie selectively acting on differentiated cells.

Accordingly, the present invention provides a method of culture of embryonic stem (ES) cells, comprising maintaining the ES cells in the presence of a compound which selectively inhibits propagation or survival of cells other than ES cells.

It is an advantage that the invention uses a compound that acts on differentiated cells and enables selective removal or killing or retardation of growth of cells that have differentiated, thus facilitating retention of a relatively pure culture of ES cells. Previously, use of known ES propagating factors, say, LIF, resulted in a certain low level of differentiation of ES cells. This level can further be reduced according to the invention, using both the known factor and the compound of the invention.

The effect of the compound of the invention is selective in that its inhibitory effects are seen to a greater extent on differentiated cells than on ES cells. It is preferred that the compound has substantially no inhibitory effect on ES cells.

Preferably, the compound inhibits a signalling pathway which when activated or at least maintained, ie not inhibited, leads to or enables propagation of cells other than ES cells. In this way the compound is effectively selectively toxic to the differentiated cells compared with its effect, if any, on ES cells. Differentiated cells either die or have their growth slowed in its presence. Inhibition may be total or partial and may occur at different points along the pathway and any compound that has the effect of inhibiting the pathway is to be regarded as an inhibitor. By reference to signalling pathway it is intended to include pathways in which an endogenous or exogenous substance has a direct effect upon cell function, e.g. propagation, cell division, the cell cycle, metabolism, as well as via an indirect effect such as via receptor-mediated signalling pathways.

A further advantage of the invention is that selection of ES cells and their maintenance in culture is achieved without the need for genetic manipulation of the cells. This represents a major advantage in particular in connection with derivation of ES cells from humans. Instead, in preferred embodiments of the invention selection and/or derivation of ES cells is achieved by the discovery of a signalling pathway, which pathway can be

blocked or inhibited, activation or maintenance of which is essential for propagation of differentiated cells but not for propagation of ES cells. By "essential" it is meant that the differentiated cell is at least severely handicapped, such as having its growth severely retarded or other fundamental cellular functions severely adversely affected, when the pathway is inhibited as well as meaning that cellular growth stops or the cell dies when the pathway is inhibited. If inhibition of the pathway did not actually result in death of the differentiated cell but instead a relative growth slowing such as to provide selective propagation of ES cells in culture then the effect desired in the invention is achieved. Preferably, though, differentiated cells have their growth substantially stopped compared with ES cells.

In an embodiment of the invention, the compound inhibits or reduces activity of a component of the ras/MAPK cascade. In use, the inhibitor is present in culture medium at non-toxic levels and inhibits a receptor-mediated pathway which would normally when activated lead to propagation of differentiated cells but which is not needed for propagation of ES cells. As a result, selective proliferation of ES cells is obtained.

In a particular embodiment of the invention, the compound inhibits one or more mitogen activated protein kinases, for example ERK1 and ERK2. In another embodiment of the invention, the compound inhibits SHP-2, for example by inhibiting binding of the enzyme to gp130, having a similar effect. In a further embodiment, the inhibitor inhibits MEK.

In a specific embodiment of the invention, described in examples below, the MEK inhibitor PD098059 is used to sustain ES cells in culture in an undifferentiated state. A further specific embodiment of the invention is the mitogen-activated protein kinase kinase inhibitor U0126, which is selective for MEK-1 and MEK-2 (Favata et al, 1998). Anthrax lethal factor has also been found to exhibit an MAPKK inhibitory profile similar to that of

PD098059 (Duesbery et al, 1999). These various compounds may be used alone or in combination or with other factors.

The inhibitor of the invention may inhibit the cell cycle in differentiated cells, thereby preventing or slowing cell growth. A specific inhibitor of the invention inhibits MEK and induction of cyclin downstream of this enzyme is as a result disrupted; thus the various phases of the cell cycle are affected, and cyclin dependent entry into S-phase of the differentiated cells is inhibited. The selective propagation of the invention may alternatively be achieved by downregulation of a component of the ras/MAPK cascade. MKP-3 is an example of a MAP kinase phosphatase and a known downregulator of the ERKs, and in a specific example of the invention MKP-3 has been introduced into an ES cell by way of a transgene. It is further an option for selective culture of ES cells to be obtained through the use of a combination of any two or more of the inhibitors of the invention. Specifically, both a component of the ras/MAPK cascade and SHP-2 may be inhibited concurrently, though generally any combination of inhibitors is encompassed by the invention.

By "propagation", and corresponding terms, it is intended to mean that an ES cell has formed daughter cells so that the total number of ES cells is increased, ie the ES cell has survived and multiplied. "Proliferation", and its corresponding terms, is intended to have the same meaning. "Self-renewal", and its corresponding terms, is intended to mean that at least one daughter cell is identical to the parent.

According to a second aspect of the invention there is provided a method of culture of ES cells comprising maintaining ES cells in the presence of a first compound that promotes proliferation of ES cells and a second compound that enhances the response of the cells to the first compound.

This has the advantage that increased ES cell proliferation is achieved for

a given amount of the first compound. In an example of the method, LIF is used to promote ES cell propagation as is known in the art and an amount of the second compound increases the effects of LIF. The first compound preferably acts through a cell-surface receptor and exerts its activity through at least one receptor subunit, and the second compound modifies an intracellular signalling pathway so as to increase the response of the ES cell to the first compound.

The second compound may suitably be an inhibitor according to the first aspect of the invention. The second compound may also bind to or otherwise affect the same receptor subunit that transduces the ES cell growth inducing signal from the first compound.

A third aspect of the invention provides a method of culture of ES cells comprising maintaining ES cells in the presence of:-

(a) a compound that promotes propagation or survival of ES cells; and

(b) a compound that inhibits propagation or survival of cells other than ES cells.

Compound (a) may be selected from known compounds that promote ES cell proliferation, especially LIF, and used in combination with a compound (b) which may in turn be selected from compounds of the first aspect of the invention.

In a preferred embodiment of the invention the combination of compounds (a) and (b) is synergistic. Thus, ES cells are maintained in the presence of two factors that together increase the percentage of ES cells that self renew, and this increase is greater than the combined increase in percentage of self renewal when the two compounds are used in separate cultures.

Compound (b) suitably selectively inhibits a signalling pathway essential to propagation of cells other than ES cells, leading to selective death or growth inhibition of differentiated cells combined with propagation of remaining ES cells as mediated by compound (a). In an embodiment of the second aspect of the invention compound (b) is selected from the inhibitory compounds described above and below in relation to the first aspect of the invention, and is preferably an inhibitor of the ras/MAPK cascade. Compound (a) is typically selected from known compounds that promote proliferation of undifferentiated ES cells, such as a cytokine that activates the cytokine receptor gp130 in ES cells. LIF is one example. Another is a combination of IL-6 and sIL-6R.

The invention additionally provides in a fourth aspect a culture medium for culture of ES cells and comprising a compound that selectively inhibits propagation or survival of cells other than ES cells.

In a fifth aspect the invention provides a medium for culture of ES cells comprising a first compound that promotes proliferation or survival of ES cells and a second compound that enhances the response of the ES cells to the first compound.

In a sixth aspect the invention provides a culture medium for culture of ES cells and comprising (a) a compound that promotes proliferation or survival of ES cells, and (b) a compound that inhibits propagation or survival of cells other than ES cells.

The culture media of the invention preferably are characterised by the components as described in relation to the first to third aspects of the invention and preferably further comprise conventional culture media ingredients.

The invention still further provides a method of obtaining and/or

maintaining a substantially pure culture of ES cells comprising culturing ES cells in the presence of culture medium according to any of the fourth to sixth aspect of the invention. The invention is of application without limitation to ES cell type, and may suitably be applied to vertebrate cells, in particular mammalian cells, primate cells, rodent cells, and human cells. In a specific embodiment described below in further detail, mouse cells have been used. By "ES" cells it is intended to encompass embryonic stem cells, embryonic carcinoma cells, embryonic gonadal cells, embryo-derived pluripotential stem cells and germline-derived stem cells.

A yet further aspect of the invention provides a method of deriving ES cells comprising isolating cells from an embryo or embryoid body and maintaining a culture of those cells in the presence of a compound that selectively inhibits propagation or survival of cells other than ES cells. The cells thereby obtained may then be maintained in the presence of the or a further compound that selectively inhibits propagation or survival of cells other than ES cells. In a particular embodiment described below, the method comprises developing an embryo *in vivo*, harvesting the embryo prior to pro-amniotic cavity formation and isolating cells therefrom, and deriving ES cells from the isolated cells or, alternatively, culturing the embryo *in vivo* in the presence of the compound prior to isolating ES cells therefrom.

A yet still further aspect of the invention provides a method of deriving ES cells comprising developing an embryo *in vitro*, isolating cells from the inner cell mass of the embryo and maintaining those cells in the presence of a compound that selectively inhibits propagation or survival of cells other than ES cells. The method may comprise removing primitive endoderm prior to culture in the presence of the compound.

In the above two aspects of the invention relating to derivation of ES cells, the compound that selectively inhibits propagation or survival of cells other

than ES cells is selected from compounds which inhibit activity of the enzyme SHP-2, inhibit the ras/MAPK cascade, inhibit MEK, inhibit a mitogen activated protein kinase or inhibit cyclin dependent entry into S-phase of non-ES cells. Thus is opened the possibility to derive ES cells through the ES cell - selective mechanisms of the invention, to derive ES cells for the first time for various species from which this has hitherto not been possible.

In a specific embodiment of the present invention, ES cells were propagated *in vitro* whilst retaining the capacity, upon reintroduction into a blastocyst, to contribute to all cell types of the developing animal, including the germ line. These cells therefore represent a tractable experimental system with which to study the regulation of stem cell self-renewal. The identification of underlying mechanisms that regulate ES cell propagation should allow the development of improved strategies for establishing ES cell lines from other mammalian species and may contribute to our understanding of self renewal in somatic stem cells.

Ligand mediated engagement of gp130 in differentiated cells results in the recruitment and phosphorylation of the STAT3 and SHP-2 signalling molecules. Stimulation of ES cells through either endogenous gp130 or the chimeric GRgp(278) receptor increased the tyrosine phosphorylation of SHP-2. This modification was blocked when tyrosine 118 of gp130 was mutated to phenylalanine, confirming that this single tyrosine is essential for recruitment of SHP-2. In spite of this deficit, the mutated receptor was fully capable of directing self-renewal of ES cells, proving effective even at low concentrations of activating ligand. Interestingly, ES cells in which both copies of the endogenous SHP-2 gene have been mutated are viable and undergo self-renewal. Since the mutant protein carries a deletion of the N-terminal SH2 domain, a region which is known to bind to gp130 these ES cells might be expected to show an altered responsiveness to LIF - in fact, the cells exhibited impaired capacity to differentiate in vitro.

Since activation of ERK 1 and ERK2 is also associated with stimulation of gp130 in ES cells, we examined the activation of these MAP kinases by the chimeric receptors. Whereas stimulation of gp130 in ES cells triggered an increase in ERK1 and ERK2 phosphorylation, no response could be detected upon engagement of the Y118F chimeric receptor. Furthermore, treatment of ES cells with inhibitory concentrations of the MEK inhibitor PD098059 did not block but rather appeared to slightly enhance self-renewal of ES cells. Notably, however, in the absence of LIF, PD098059 did not prevent ES cell differentiation. These results establish that, in accordance with the invention, the activation of ERK1 and ERK2, via either SHP-2 or another pathway such as Shc, is not critical in maintaining the proliferation of ES cells. Selective propagation of ES cells is obtainable due to the relatively greater importance of activation of these enzymes in non-ES cells.

This lack of a requirement for gp130-dependent ERK activation may be related to the quasi-transformed nature of ES cells. An established function of ERKs in differentiated cells is to regulate the transition through G1/S, at least in part through the induction of cyclin D. However, ES have a very short G1 phase and appear to posses few of the G1 associated control mechanisms (Savatier et al., 1994; Savatier et al., 1996). Furthermore, the reduced dependence on ERK signalling is consistent with the observation that ES cells continue to proliferate in the absence of serum, a powerful mitogen and inducer of ERK activity (Johansson and Wiles, 1995). In this way, inhibition of cyclin-dependent entry into S-phase in accordance with the invention enables selective propagation and/or survival of ES cells.

In vitro differentiation of ES cells is associated with the induction of G1 cyclin expression, the establishment of a long G1 phase and a decrease in the rate of cell division (Savatier et al., 1996). This transition probably reflects the changes that normally occur in the embryo at gastrulation, after the initial rapid expansion of epiblast cells. Interestingly, inhibition of SHP-2 activity during embryonic development of either xenopus or mice is

associated with failure to gastrulate normally with defects in the formation of mesodermal cell lineages. The differentiation of epiblast cells may therefore represent the point at which embryonic cells first become subservient to normal growth control mechanisms. Significantly, this is also the stage at which transplanted grafts of embryonic tissue lose the capacity to form malignant teratocarcinomas.

SHP-2/ERK activation is thus not essential for ES cell proliferation. Our results suggest that tyrosine 118 downregulates the activity of the gp130 receptor, causing a dramatic shift in dose response and prolonged activation of STAT3 in cells stimulated via the GRgp(Y118F) receptor. Until recently, SHP-2 had primarily been regarded as positive effector of signalling, either as an adaptor protein or a potential activator of src family kinases. However, a negative regulatory function was suggested both by its homology to SHP-1, a suppresser of erythropoietin receptor function, and its interaction with CTLA-4, an inhibitor of the T cell receptor. It has been reported recently that mutation of tyrosine 118 increases STAT3 signalling in neuroblastoma and hepatoma cells. Transcription from STAT3 responsive promoter constructs was also increased by overexpression of catalytically inactive SHP-2 proteins, pointing to the phosphatase as a likely mediator of this effect. This conclusion was supported by sustained phosphorylation of both the Y118F receptor and its associated JAK kinases. However, overexpression of catalytically inactive SHP-2 proteins produced only a slight shift in dose response in transfected ES cells (TB, CS unpublished), indicating that loss of phosphatase activity of SHP-2 may not be wholly responsible for the increased activity of the GRgp(Y118F) receptor.

The non-essential function of gp130-dependent SHP-2 and ERK activation in ES cells further emphasises the pre-eminent role of STAT3 in self-renewal. However, the restricted growth of GRgp(Y118F) transfectants in high concentrations of G-CSF is intriguing since it indicates that

excessive signalling can interfere with ES cell growth. Hyperactivation of STAT3 is implicated in this phenotype because stimulation of the Y118F receptor results in prolonged tyrosine phosphorylation of STAT3 and sustained activation of an endogenous STAT3 target gene SOCS3. Furthermore, combined mutagenesis of some of the STAT3 docking sites can suppress the effect of the Y118F substitution.

Signals further downstream of tyrosine 118 could also influence the growth and differentiation of ES cells. The increase in self-renewal observed on treatment with the MEK inhibitor PD098059 implies that ERK activation impairs the propagation of ES cells. Interestingly, studies of gp130-dependent regulation of PC12 cell and astrocyte differentiation have suggested that activation of the MAPK pathway may antagonize signals mediated via STAT3. In both cases, reduced MAPK activity resulted in augmented transcription from STAT3 dependent reporter constructs. In ES cells treated with PD098059, self-renewal was enhanced even at saturating levels of LIF. This suggests that the effect of the inhibitors of the invention is not simply due to an inhibition of ERK activity stimulated by LIF but may occur by blocking the actions of differentiation inducers present in serum, or those secreted by ES cells and their differentiated progeny.

The present invention is now described in specific embodiments illustrated by drawings in which:-

Fig. 1 shows gp130-dependent phosphorylation of SHP-2 in ES cells;

Fig. 2 shows effect of mutating tyrosine 118 on gp130-dependent self-renewal and growth of ES cells;

Fig. 3 shows gp130-dependent phosphorylation of ERK1 and ERK2 in ES cells;

Fig. 4 shows effect of the MEK inhibitor, PD098059, on ES cell self-renewal and ERK activation;

Fig. 5 shows effect of PD098059 on ES cell pluripotency;

Fig. 6 shows decay of activated STAT3 following stimulation of gp130 and the chimeric GRgp130 receptors;

Fig. 7 shows gp130-dependent induction of SOCS-3 gene expression in ES cells; and

Fig. 8 shows the effect of an MEK inhibitor on stem cell differentiation.

In more detail, Fig.1 shows gp130-dependent phosphorylation of SHP-2 in ES cells. ES cells expressing either the GRgp(278) or GRgp(Y118F) chimeric receptors were induced with IL-6 (100 ng/ml plus sIL-6R) or G-CSF (30 ng/ml) for 15 minutes. SHP-2 protein was immunoprecipitated from lysates of unstimulated or stimulated cells, fractionated on a SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. The filter was probed with anti-phosphotyrosine antibody (upper panel), stripped and reprobed with anti-SHP-2 antibody (lower panel). The position of tyrosine phosphorylated forms of SHP-2 and two additional proteins are indicated by arrows.

Fig.2 shows the effect of mutating tyrosine 118 on gp130-dependent self-renewal and growth of ES cells:

(A) Stem cell renewal mediated by GRgp(278) and GRgp(Y118F) chimeric receptors in response to G-CSF. Self-renewal, as measured by β-galactosidase expression from the *Oct-4* locus was assayed after 6 days in culture with G-CSF (300 fg-30 ng/ml). Data for two independent clones are represented as means ± s.e.m. for duplicate determinations of triplicate samples normalized relative to the response with IL-6(100 ng/ml plus sIL-6R).

(B) Photomicrographs of X-gal stained, representative colonies formed by GRgp(278) and GRgp(Y118F) transfectants after 6 days culture with 300 fg, 30 pg and 30 ng/ml of G-CSF.

(C) Photomicrographs of representative colonies formed by GRgp(278) and GRgp(Y118F) transfectants after 6 days culture with no

cytokine, IL-6 (100 ng/ml + sIL-6R), G-CSF(30 ng/ml) or , IL-6 (100 ng/ml + sIL-6R) and G-CSF(30 ng/ml).

Fig.3 shows gp130-dependent phosphorylation of ERK1 and ERK2 in ES cells. ES cells expressing either the GRgp(278) or GRgp(Y118F) chimeric receptors were either untreated or stimulated with IL-6(100 ng/ml plus sIL-6R) or G-CSF (30 ng/ml) for 10 or 20 minutes. Cell lysates were separated on a 10% SDS-acrylamide gel, electroblotted onto a nitrocellulose membrane and probed sequentially with antibodies specific for the active phosphorylated form of ERK and STAT3. Reprobing the stripped filter with an antibody that binds to both phosphorylated and dephosphorylated ERKs verified that equivalent amounts of protein were loaded in the samples.

Fig.4 shows the effect of the MEK inhibitor, PD098059, on ES cell self-renewal and ERK activation:

(A) Self-renewal of ES cells treated with PD098059. D027 ES cells grown at a subsaturating level of LIF (5 U/ml) were treated with PD098059 for 5 days and assayed for β-galactosidase expression from the *Oct-4* locus. Data are means ± s.e.m for duplicate determinations of triplicate samples normalized relative to the response to LIF.

(B) PD098059 dependent inhibition of ERK activation. GRgp(278) transfected D027 cells were cultured with a subsaturating level of LIF (5 U/ml) and PD098059 for 48 hours. Cells were then stimulated with G-CSF (30 ng/ml) for 10 minutes, lysed in sample buffer and analyzed for ERK activation by immunoblotting with phosphospecific anti-ERK antibodies. Subsequent probing of the filter with an antibody that binds to both phosphorylated and dephosphorylated ERKs confirmed that equivalent amounts of protein were loaded in all samples.

(C) Effect of PD098059 on the dose response of ES cells to LIF. The dose response of D027 ES cells to LIF in 25 μM PD098059 or vehicle (0.05% DMSO) was measured by β-galactosidase expression from the *Oct-4* locus. Data are means ± s.e.m for duplicate determinations of triplicate

samples normalized relative to the maximum response of cells to treatment with LIF(111 U/ml) plus vehicle.

Fig. 5 shows the effect of PD098059 on ES cell pluripotency. ZIN40 ES cells were treated with 25 μM PD098059 plus 5 U/ml LIF for 48 hours, re-fed with medium containing LIF for a further 24 hours and then microinjected into C57BL/6 blastocysts. Embryos were collected at day 9.5 of pregnancy and stained for β-galactosidase activity. Representative embryos are shown in the panel.

Fig. 6 shows decay of activated STAT3 following stimulation of gp130 and the chimeric GRgp130 receptors. GRgp(278) and GRgp(Y118F) ES cell transfectants were stimulated with IL-6 (100 ng/ml plus sIL-6R) or G-CSF (30 ng/ml) for 25 minutes (0*), re-fed with cytokine free medium and samples were collected at 40 minute intervals. Immunoblots of cell lysates were probed sequentially with an antibody specific for the active phosphorylated form of STAT3 and then with an antibody that recognizes both phosphorylated and unphosphorylated STAT3. Note that activation of STAT3 is associated with the appearance of a slower migrating STAT3 species, presumed to be the serine phosphorylated form of STAT3.

Fig.7 shows gp130-dependent induction of *SOCS-3* gene expression in ES cells.

(A) Northern analysis was performed on total RNA (10 g) prepared from ES cells expressing the GRgp(278), GRgp(Y126-275F) and GRgp(Y118F) receptors, unstimulated (-) or stimulated either with LIF (L, 100 units/ml) or G-CSF (G, 30 ng/ml) for the indicated times (minutes). Hybridisation of the $\approx$ 3 kb SOCS-3 mRNA and ethidium bromide staining of the 18S rRNA is shown in the upper and lower three panels, respectively.

(B) Graphic representation of SOCS-3 mRNA expression shown in panel A. SOCS-3 mRNA hybridization was quantitated by phosphorimage

analysis and signals were normalized relative to those obtained in each cell line at 90 minutes after stimulation with LIF.

Fig. 8 shows that an MEK inhibitor of the invention sustains undifferentiated ES cells in an aggregate culture.

Example 1

Materials and Methods

Cell culture and Transfection

ES cells were maintained without feeder cells in Glasgow modification of Eagles medium (GMEM) containing 10 % fetal calf serum, 0.1 mM 2-mercaptoethanol and LIF. D027 cells have both copies of the *lif* gene inactivated by homologous recombination and an IRES-βgeo reporter gene inserted within the *Oct-4* gene locus. ZIN40 cells carry a nuclear localised β-galactosidase marker gene that is widely expressed in differentiated cell types. For transfections, 2 x10^7 cells were electroporated with 100 μg of linearised plasmid DNA at 0.8kV and 3 μF in a 0.4 cm cuvette using a Bio-Rad gene pulser. Stably transfected clones were selected in medium containing 20 μg/ml zeocin (Invitrogen).

Plasmid construction

The GR/gp130 chimeric receptors were generated by fusing the extracellular domain of the human G-CSFR to an EcoRI fragment of mouse gp130 containing the transmembrane domain and the entire cytoplasmic region. The phenylalanine substitution of tyrosine 118 was introduced into the intracellular domain of gp130 by PCR overlap mutagenesis. The PCR product was substituted into the GRgp(278) chimaera and sequenced. The receptor cDNAs were inserted within expression vector pCAGIZ. This vector contains a bicistronic expression cassette, consisting of cytomegalovirus enhancer-human β-actin promoter, a site for insertion of the receptor cDNA, an internal ribosome entry site (IRES) and the zeocin

resistance gene.

Self-renewal assay

Expression of β-galactosidase from the *Oct-4* locus in D027 cells was quantitated in an ONPG assay. Cells were plated at 5000 per well in a 24-well dishes and cultured for 6 days in the presence or absence of cytokine. When cells were treated with PD098059, they were plated at 2500 per well and cultured overnight in normal growth medium prior to addition of the inhibitor. On day 6, cells were washed once with PBS and lysed in 0.4 ml of 0.25 M Tris pH 7.5, 0.5 mM DTT, 0.5 % NP40. Lysate (40 μl) was mixed with 100 μl of ONPG buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgCl_2$, 50 mM 2-mercaptoethanol, 1.2 mM ONPG) in a microtitre plate, incubated at 37° C for 2-4 hours and the absorbance was read at 420 nm. All assays were performed in triplicate.

Immunoprecipitation and Immunoblotting

One day after plating (2-3 x 10^6 cells per 100 mm dish), ES cells were re-fed with medium containing 1 % foetal calf serum and lacking cytokines. The following day, cells were transferred to serum free medium for 4 hours prior to stimulation with IL-6 (100 ng/ml plus soluble receptor) or G-CSF (30 ng/ml) for 15 minutes. Cells were then washed once with ice-cold PBS and scraped off in 1 ml of ice-cold lysis buffer (150 mM NaCl, 10 mM Tris.HCl pH 7.4, 0.5 % NP40, 1 mM $NaVO_3$, 1mM EDTA, 0.5 mM PMSF). Cleared lysates were incubated with 1 μg anti SHP-2 antibody (Santa Cruz) at 4° C for 1 hour and then protein A sepharose was added and the incubation continued overnight. Immunoprecipitates were lysed in 2 x SDS sample buffer, fractionated by electrophoresis on a 10 % SDS polyacrylamide gel and electroblotted onto nitrocellulose. After overnight treatment with blocking buffer (25 mM Tris-HCl pH 7.4, 2.7 mM KCl, 140 mM NaCl, 0.1 % Tween 20, 1 % BSA) the membranes were probed sequentially with anti-phosphotyrosine antibody 4G10 (Transduction Laboratories) and anti-SHP-2 antibody. Blots were incubated with horse

radish peroxidase coupled anti-rabbit IgG and developed using ECL reagents (Amersham). Antibodies were stripped from the membranes between probings by incubation at 50° C for 30 minutes in 62.5 mM Tris.HCl pH 6.8, 2 % SDS, 100 mM 2-mercaptoethanol.

For analysis of STAT3 and ERK phosphorylation, 1 x 10^6 ES cells were plated per well of 6-well dishes. Cells were serum starved and treated with cytokines as described above and then lysed in 100 μl SDS sample buffer. Ten microlitre aliquots were fractionated on a 10 % SDS polyacrylamide gel, electroblotted onto nitrocellulose and probed with anti-ERK and anti-STAT3 antibodies according to the directions provided by supplier (New England Biolabs).

Northern Blotting

RNA was prepared as described, except that the second iso-propanol precipitation was replaced by an overnight precipitation at 4° C in 2 M LiCl to remove contaminating DNA. Total RNA (10 μg) was separated on a 0.66 M formaldehyde/agarose gel and transferred to a nylon membrane (Boehringer). Hybridisation was performed as described, using a [α-32P]dCTP labelled DNA probe. The probe was an EcoRI-NotI fragment of a SOCS3 EST plasmid (IMAGE clone number 864805, Genbank accession number AA444828 obtained from HGMP). Sequence analysis verified that the probe corresponded to nt 1666-2176 of SOCS-3 (U88328).

Chimaera analysis

ZIN40 ES cells were injected into C57BL/6 blastocysts and transferred into pseudopregnant mice. Mice were sacrificed at day 9.5 of pregnancy and the embryos were stained with X-gal.

Results

Tyrosine 118 is required for gp130-dependent phosphorylation of SHP-2 in ES cells

Previous studies in BAF pro-B cell lines have shown that gp130-dependent activation of SHP-2 and the MAPKs, ERK1 and ERK2, is mediated via tyrosine 118 (located 118 amino acid residues from the membrane) in the cytoplasmic region of gp130. To examine the functional role of tyrosine 118 in ES cells, we constructed cDNAs encoding chimaeric receptors consisting of the extracellular domain of the granulocyte colony stimulating factor receptor (G-CSFR) fused to the transmembrane and cytoplasmic region of gp130. Since ES cells do not normally express the G-CSFR and show no self-renewal response to G-CSF (data not shown), these chimaeric receptors can be used to examine signaling independently of endogenous cytokine receptors. cDNAs encoding either the unmodified chimaeric receptor, GRgp(278), or a mutated receptor in which phenylalanine had been substituted for tyrosine 118, GRgp(Y118F), were cloned into the pCAGIZ expression vector and stably introduced into D027 ES cells by electroporation.

In several differentiated cell types, SHP-2 becomes tyrosine phosphorylated following its recruitment to a tyrosine phosphorylated gp130 receptor subunit. To examine whether SHP-2 undergoes this modification in ES cells, SHP-2 immunoprecipitates were prepared from GRgp(278) and GRgp(Y118F) transfectants following stimulation with either IL-6 (plus sIL-6R) or G-CSF and probed for phosphotyrosine by western blotting (Figure 1A). An increase in phosphorylated SHP-2 was detected in cells stimulated through either the endogenous gp130 or the GRgp(278) receptors. Two additional tyrosine phosphoproteins co-precipitated with the phosphorylated SHP-2. The band migrating at approximately 100 kD may represent Gab1, the IRS-1-related adaptor protein previously reported to associate with phosphorylated SHP-2. No increase in phosphorylation of SHP-2 was detected following stimulation of the GRgp(Y118F) receptor, confirming that tyrosine 118 is essential for effective gp130-dependent phosphorylation of this phosphatase in ES cells.

SHP-2 activation is not required for ES cell self-renewal

In order to determine whether activation of SHP-2 is necessary for the propagation of ES cells, the response of GRgp130 transfectants to G-CSF was measured in a self-renewal assay. D027 cells have a *LacZ* gene inserted within the stem cell specific gene, *Oct-4*. As a consequence, expression of this integrated reporter gene is restricted to undifferentiated ES cells and the resulting β-galactosidase activity provides a measure of stem cell self-renewal. In addition, both copies of the LIF gene have been inactivated through gene targeting, thus reducing autocrine stimulation of ES cell growth. β-galactosidase activity from two independently isolated clones for each receptor construct was measured in medium density cultures after 6 days of treatment with 300fg-30ng/ml G-CSF.

The data presented in Figure 2A shows that self-renewal of GRgp(278) transfectants increased in a dose dependent manner, reaching a plateau at 3-30 ng/ml G-CSF. In contrast, the maximal self-renewal response of GRgp(Y118F) ES cells was achieved at just 30 pg/ml G-CSF. The morphology of the GRgp(Y118F) colonies maintained in 30 pg/ml G-CSF was typical of undifferentiated ES cells (Figure 2B). This result establishes that activation of SHP-2 through tyrosine 118 is not required to direct ES cell self-renewal. Equivalent levels of both receptor chimaeras were expressed at the cell surface of ES cell transfectants, as judged by binding studies with ^{125}I-labelled G-CSF (data not shown). Therefore the shift in dose response suggests that the mutant receptor may have enhanced signaling activity.

Interestingly, at higher concentrations of G-CSF, GRgp(Y118F) transfectants formed small aggregates of cells rather than the more flattened colony morphology normally associated with undifferentiated ES cells (Figure 2B). These colonies expressed β-galactosidase and stained positive for the stem cell marker alkaline phosphatase (Figure 2B and data not shown), indicating that the ES cells remained undifferentiated. This was

confirmed by the resumption of typical ES cell growth and colony morphology when, following the initial treatment with G-CSF, these cultures were refed with medium containing IL-6 plus sIL-6R (data not shown).

The unusual appearance of GRgp(Y118F) cells in high concentrations of G-CSF is unlikely to be simply due to an increase in affinity of the Y118F receptor for G-CSF because this response is not observed in wild type cells treated with high levels of LIF, or in GRgp(278) transfectants treated with saturating levels of IL-6(plus sIL-6R), G-CSF, or IL-6(plus sIL-6R) plus G-CSF(Figure 2C). Furthermore the phenotype of GRgp(Y118F) cells in high levels of G-CSF was maintained when cells were simultaneously stimulated with G-CSF and IL-6 (plus sIL-6R). This observation excludes the explanation that the unusual ES cell morphology is due to a partial loss of self-renewal signals and suggests that the phenotype arises from hyperactivation of signals downstream of gp130. Collectively these data point to a key role for tyrosine 118 in downregulating gp130 signaling in ES cells.

Tyrosine 118 is necessary for activation of ERK1 and ERK2

Since activation of SHP-2 may couple gp130 to the ERK pathway, we examined whether tyrosine 118 was also required for activation of ERK1 and ERK2 in ES cells. Activation of ERKs in GRgp130 transfectants treated with G-CSF or IL-6 (plus sIL-6R) was assessed by immunoblotting with an antibody specific for the phosphorylated (activated) forms of ERK1 and ERK2 (Figure 3). Basal levels of activated ERK were consistently detected in untreated cells following serum starvation. Increased ERK phosphorylation was observed in cells stimulated via the endogenous gp130 and GRgp(278) receptors. This was not evident on stimulation through the GRgp(Y118F) chimaera. Reprobing with an antibody specific for the tyrosine phosphorylated form of STAT3, confirmed that both chimaeric receptors were effective at activating STAT3. These results

establish that tyrosine 118 mediates activation of the ERK pathway in ES cells.

Blocking ERK activation with PD098059 does not impair ES cell propagation

The capacity of GRgp(Y118F) to signal self-renewal implies that ERK activation is not required for the propagation of ES cells. To test this hypothesis, D027 cells were cultured in the presence of the specific MEK inhibitor, PD098059. A sub-saturating concentration of LIF (5 U/ml) was used in these experiments to increase the sensitivity of the assay to changes in self-renewal signaling. Surprisingly, treatment of ES cells with 3-25 μM PD098059 did not inhibit self-renewal when compared with cells cultured in vehicle alone (Figure 4A). More surprisingly, in fact, the level of self-renewal increased in a dose dependent manner with the maximum level being achieved at 12-25 μM. At concentrations greater than 50 μM PD098059 the growth of ES cells was impaired, possibly as a result of some non-specific inhibitory effect of the drug, resulting in small undifferentiated colonies which stained positive for β-galactosidase by X-gal staining (data not shown).

To verify that ERK activation through gp130 was continuously suppressed by PD098059 in these long-term cultures, GRgp(278) cells were incubated for 48 hours with the inhibitor plus LIF and then stimulated through the chimaeric receptor with G-CSF. The immunoblot revealed that G-CSF dependent phosphorylation of ERK1 and ERK2 was progressively reduced from 3-12 μM, and effectively blocked at 25 μM PD098059 (Figure 4B). The continued proliferation of undifferentiated ES cells at inhibitory concentrations of PD098059 confirms that gp130-dependent activation of ERK1 and ERK2 is not required for the propagation of ES cells.

The effect of PD098059 on self-renewal suggested that the inhibitor might alter the dose response of ES cells to LIF. Self-renewal of ES cells was

assayed following treatment with 0.1-100 U/ml LIF either in the presence of 25 μM PD098059 or vehicle (0.2 % DMSO)(Figure 4C). Treatment with PD098059 increased the level of β-galactosidase activity at all concentrations of LIF. This implies that the drug does not alter the dose dependency of ES cells but rather enhances their response to LIF. Significantly, PD098059 did not block the differentiation of ES cells in the absence of LIF.

ES cells propagated in PD098059 remain pluripotent

ES cell colony morphology and Oct-4 expression are reliable indicators of the undifferentiated phenotype, but do not establish that the cells are pluripotent. We therefore determined whether ES cells propagated in the absence of gp130-dependent ERK signaling have the capacity to incorporate into the developing embryo and differentiate appropriately. Cells were cultured at low density (1000 cells/cm^2) for 48 hours in the presence of LIF plus 25 μM PD098059, or in the absence of LIF. They were then re-fed with medium containing LIF but lacking the inhibitor for a further 24 hours before microinjection into mouse blastocysts. ZIN40 cells were used in this experiment, since they carry a nuclear localised β-galactosidase marker that is widely expressed in differentiated cell types. Staining of mid-gestation embryos for β-galactosidase revealed that ES cells treated with PD098059 contributed to chimaeras (Figure 5). However, cells cultured in the absence of LIF for 48 hours were incapable of colonizing the embryo (data not shown). This result confirms that gp130-dependent ERK activity is not required for maintaining the pluripotency of ES cells.

Attenuation of the STAT3 signal is mediated via tyrosine 118

We have previously established that activation of STAT3 is essential for gp130-dependent self-renewal of ES cells. To determine whether mutating tyrosine 118 affects this key regulator, activation of STAT3 was compared in GRgp(278) and (Y118F) transfectants. The acute stimulation of cells for 25 minutes with 30 fg/ml to 300 ng/ml of G-CSF did not reveal a

significant difference between the levels of tyrosine phosphorylation of STAT3 induced by the chimaeric receptors (data not shown). However, signaling through the receptors was distinguished when the duration of the STAT3 signal was examined (Figure 6). Cells were stimulated with either G-CSF or IL-6 (plus sIL-6R) for 25 minutes, re-fed with cytokine free medium and then samples were collected at 40 minute intervals. A similar time course for the decay of phosphorylated STAT3 was obtained following stimulation through either the endogenous gp130 receptor or GRgp(278), with the signal being undetectable at 120 minutes. In contrast, the activation of STAT3 was sustained in G-CSF treated GRgp(Y118F) cells and could still be detected at 160 minutes. This result indicates that tyrosine 118 mediates a signal that normally attenuates the activation of STAT3.

Substitution of tyrosine 118 leads to hyperinduction of a chromosomal target gene

In order to investigate whether the prolonged activation of STAT3 influenced gene regulation in ES cells, we examined the expression of *SOCS* genes. These genes are rapidly induced by cytokines and encode proteins that can function as negative regulators of cytokine receptor function. SOCS-1 is a STAT3 target in M1 cells but this may not be the case in ES cells as we have not observed any increase in SOCS-1 expression in response to LIF (data not shown). In contrast, expression of SOCS-3 was transiently induced in ES cells stimulated either through the LIFR/gp130 complex with LIF or through the GRgp(278) chimaera (Figure 7). The peak level of observed expression occurred at 90 minutes after addition of cytokine, and returned close to uninduced levels by 3 hours. There was no induction of SOCS-3 transcripts in ES cells stimulated through a chimaeric receptor, GRgp(Y126-275F), in which the four STAT3 docking sites have been eliminated by site directed mutagenesis. This result implies that the *SOCS-3* gene is a target for STAT3 in ES cells. Significantly, following activation of the GRgp(Y118F) receptor the peak

level of SOCS-3 expression obtained at 90 minutes was enhanced and in contrast to stimulation through the LIF receptor or GRgp(278), SOCS-3 mRNA levels remained elevated until at least 6 hours post-stimulation. It seems likely therefore that the prolonged activation of STAT3 results in enhanced expression of its target genes. This may underlie the shift in dose response to G-CSF observed for GRgp(Y118F) transfectants.

MEK Inhibitor PD089059 sustains undifferentiated ES cells in aggregate culture.

Aggregation induces ES cells to differentiate and form structures known as embryoid bodies that contain multiple differentiated cell types. Undifferentiated cells are largely or wholly eliminated during embryoid formation due to induced differentiation and/or apoptosis.

IOUD2 ES cells, which carry a targeted integration of βgeo into the *Oct4* locus were used to enable visualisation of undifferentiated cells by histochemical staining for β-galactosidase. Aggregates were formed in hanging drops by seeding 100 cells/20μl drop in the presence of 0, 25, 50, 75 or 100μm PD089059. Aggregates were maintained for 6 days, then transferred to gelatin-coated dishes and allowed to attach overnight. Cultures were then fixed and stained for β-galactosidase activity. In the absence of the Mek inhibitor, the embryoid bodies were well differentiated and very few *Oct4* β-galactosidase expressing cells were present in the outgrowths (Fig. 8, upper panel). In the presence of PD089059 however, the representation of undifferentiated β-galactosidase positive cells increased in a dose dependent fashion. At PD089059 concentrations of 75-100μm, the great majority of cells were undifferentiated (Fig. 8, lower panel). The numbers of undifferentiated cells in these conditions vastly exceeded these present in control cultures in the absence of MEK inhibitor, therefore this result is not simply due to ablation of cells This finding indicates that ERK activation is critical to the process of embryoid body differentiation and that differentiation of stem cells can be prevented by

reducing or abolishing Mek activity.

Example 2

Derivation of Stem Cell Lines

In order to isolate stem cell lines, embryos were developed *in vivo* and allowed to implant then harvested prior to pro-amniotic cavity formation (6.5dpc equivalent). Epiblasts were microdissected and placed in suspension culture in ES cell medium in the presence of PD098059. After several days in suspension culture the epiblasts were dissociated and plated on tissue culture plastic. PD098059 was maintained in the culture medium until expanding populations of undifferentiated stem cells were generated. Stem cell lines were also derived from embryos developed *in vitro* by immunosurgical isolation of the ICM at the blastocyst stage followed by microsurgical removal of the primitive endoderm and culture in ES cell medium plus PD098059.

In accordance with the invention, the independence of ES cell self-renewal from ERK activation has important practical applications. Inhibitors such as of the Ras/MAPK pathway promote the propagation of undifferentiated ES cells. By suppressing the growth and maturation of differentiated cell types, such inhibitors facilitate the routine manipulation and *de novo* derivation of ES cells.

Example 3

Expression of MKP-3 transgene

A transgene coding for MKP-3 was inserted into an ES cell and a culture of ES cells obtained therefrom expressing MKP-3. It was observed that a culture highly purified in respect of ES cells was maintained and that differentiation of these ES cells was substantially reduced compared with differentiation of ES cells in a control culture not expressing the MKP-3 transgene. This experiment also provides genetic evidence in support of the invention.

References

Dudley, D.T., L. Pang, S.J. Decker, A.J. Bridges, and A.R. Saltiel. 1995. A synthetic inhibitor of the mitogen-activated protein kinase cascade. *Proc. Natl Acad. Sci. USA*. 92:7686-7689.

Duesbery NS et al, J Appl Microbiol, August 1999, 87(2), pp289-293. Anthrax lethal factor causes proteolytic inactivation of mitogen-activated protein kinase kinase.

Favata MF, et al, J Biol Chem, 17 July 1998, 273(29), pp18623-32. Identification of a novel inhibitor of mitogen-activated protein kinase kinase.

Johansson, B.M., and M.V. Wiles. 1995. Evidence for involvement of Activin A and Bone morphogenetic protein 4 in mammalian mesoderm and hematopoietic development. *Mol. Cell. Biol.* 15:141-151.

Pages, G., P. Lenormand, G. L'Allemain, J.C. Chambard, S. Meloche, and J. Pouyssegur. 1993. Mitogen-activated protein kinases p42mapk and p44mapk are required for fibroblast proliferation. *Proc Natl Acad Sci U S A*. 90:8319-8323.

Savatier, P., S. Huang, L. Szekely, K.G. Wiman, and J. Samarut. 1994. Contrasting patterns of retinoblastoma protein expression in mouse embryonic stem cells and embryonic fibroblasts. *Oncogene*. 9:809-818.

Savatier, P., H. Lapillonne, L.A. van Grunsven, B.B. Rudkin, and J. Samarut. 1996. Withdrawal of differentiation inhibitory activity/leukemia inhibitory factor up-regulates D-type cyclins and cyclin-dependent kinase inhibitors in mouse embryonic stem cells. *Oncogene*. 12:309-322.

CLAIMS

1. A method of culture of embryonic stem (ES) cells, comprising maintaining the ES cells in the presence of a compound which selectively promotes self-renewal of the ES cells and/or inhibits propagation or survival of cells other than ES cells.

2. A method according to Claim 1 wherein the compound inhibits a cell signalling pathway and wherein maintenance or activation of the pathway is essential to propagation or survival of cells other than ES cells but is not essential to propagation or survival of ES cells.

3. A method according to Claim 1 or 2, wherein the compound inhibits cyclin dependent entry into S-phase of cells other than ES cells.

4. A method according to Claim 1, 2 or 3 wherein the compound inhibits or reduces the activity of the enzyme SHP-2.

5. A method according to any of Claims 1 to 3 wherein the compound inhibits or reduces the activity of the ras/MAPK cascade.

6. A method according to any of Claims 1 to 3 wherein the compound inhibits or reduces the activity of MEK.

7. A method according to any of Claims 1 to 3 wherein the compound inhibits or reduces the activity of a mitogen activated protein kinase.

8. A method according to any of Claims 1 to 3 comprising using a combination of at least two compounds selected from compounds that inhibit SHP-2, the ras/MAPK cascade, MEK and a mitogen activated protein kinase.

9. A method of culture of ES cells comprising maintaining ES cells in

the presence of a first compound that promotes proliferation of ES cells and a second compound that enhances the response of the cells to the first compound.

10. A method according to Claim 9, wherein the first compound acts through a cell-surface receptor and exerts its activity through at least one receptor subunit, and the second compound modifies an intracellular signalling pathway so as to increase the response of the ES cell to the first compound.

11. A method according to Claim 9 or 10, wherein the second compound is an inhibitor of the ras/MAPK cascade.

12. A method of culture of ES cells comprising maintaining a culture of ES cells in the presence of:-

(a) a compound that promotes propagation or survival of ES cells; and

(b) a compound that inhibits propagation or survival of cells other than ES cells.

13. A method according to Claim 12, wherein the combination of compounds (a) and (b) is synergistic.

14. A method according to Claim 12 or 13 wherein compound (b) selectively inhibits a signalling pathway essential to propagation or survival of cells other than ES cells.

15. A method according to any of Claims 12 to 14, wherein compound (b) is selected from compounds which inhibit activity of the enzyme SHP-2, inhibit the ras/MAPK cascade, inhibit MEK, inhibit a mitogen activated

protein kinase or inhibit cyclin dependent entry into S-phase of non-ES cells.

16. A method according to any of Claims 12 to 15 wherein compound (a) is a cytokine that activates the cytokine receptor gp130 in ES cells.

17. A method according to Claim 16, wherein the cytokine is LIF.

18. A composition for selective culture of ES cells, comprising (a) a compound that inhibits differentiation of ES cells, and (b) a synergistic amount of a compound that inhibits propagation or survival of cells other than ES cells.

19. A composition according to Claim 18 wherein compound (b) selectively inhibits a signalling pathway essential to propagation or survival of cells other than ES cells.

20. A composition according to Claim 18 or 19 wherein compound (b) is selected from compounds which inhibit activity of the enzyme SHP-2, inhibit the ras/MAPK cascade, inhibit MEK, inhibit a mitogen activated protein kinase or inhibit cyclin dependent entry into S-phase of non-ES cells.

21. A composition according to any of Claims 18 to 20 wherein compounds (a) is a cytokine that binds to the cytokine receptor gp130, such as LIF.

22. Use of a compound that selectively inhibits propagation or survival of cells other than ES cells in a method of obtaining a substantially pure culture of ES cells.

23. A culture medium for culture of ES cells and comprising a compound

that selectively inhibits propagation or survival of cells other than ES cells.

24. A culture medium according to Claim 23, comprising a compound selected from compounds which inhibit activity of the enzyme SHP-2, inhibit the ras/MAPK cascade, inhibit MEK, inhibit a mitogen activated protein kinase or inhibit cyclin dependent entry into S-phase of non-ES cells.

25. A culture medium for culture of ES cells and comprising a first compound that promotes proliferation of ES cells and a second compound that enhances the response of the ES cells to the first compound.

26. A culture medium according to Claim 25, wherein the first compound acts through a cell-surface receptor and exerts its activity through at least one receptor subunit, and the second compound modifies an intracellular signalling pathway so as to increase the response of the ES cell to the first compound.

27. A culture medium according to Claim 25 or 26 wherein the second compound is selected from compounds which inhibit activity of the enzyme SHP-2, inhibit the ras/MAPK cascade, inhibit MEK, inhibit a mitogen activated protein kinase or inhibit cyclin dependent entry into S-phase of non-ES cells.

28. A culture medium for culture of ES cells and comprising (a) a compound that promotes proliferation of ES cells, and (b) a compound that inhibits propagation or survival of cells other than ES cells.

29. A culture medium according to Claim 25, wherein the combination of compounds (a) and (b) is synergistic.

30. A culture medium according to Claim 28 or 29, wherein compound

(b) selectively inhibits a signalling pathway essential to propagation of cells other than ES cells.

31. A culture medium according to any of Claims 28 to 30, wherein compound (b) is selected from compounds which inhibit activity of the enzyme SHP-2, inhibit the ras/MAPK cascade, inhibit MEK, inhibit a mitogen activated protein kinase or inhibit cyclin dependent entry into S-phase of non-ES cells.

32. A culture medium according to any of Claims 28 to 31, wherein compound (a) is a cytokine that activates the cytokine receptor gp130 in ES cells.

33. A culture medium according to Claim 32, wherein the cytokine is LIF.

34. A method of deriving ES cells comprising isolating cells from an embryo or embryoid body and maintaining a culture of those cells in the presence of a compound that selectively inhibits propagation or survival of cells other than ES cells.

35. A method according to Claim 34 comprising dissociating cells obtained and then further maintaining the dissociated cells in the presence of the or a further compound that selectively inhibits propagation or survival of cells other than ES cells.

36. A method according to Claim 34 or 35 comprising developing an embryo *in vivo*, harvesting the embryo prior to pro-amniotic cavity formation and isolating cells therefrom.

37. A method of deriving ES cells comprising developing an embryo *in vitro*, isolating cells from the inner cell mass of the embryo and maintaining those cells in the presence of a compound that selectively inhibits

propagation or survival of cells other than ES cells.

38. A method according to Claim 37 comprising removing primitive endoderm prior to culture in the presence of the compound.

39. A method according to any of Claims 34 to 38, wherein the compound that selectively inhibits propagation or survival of cells other than ES cells is selected from compounds which inhibit activity of the enzyme SHP-2, inhibit the ras/MAPK cascade, inhibit MEK, inhibit a mitogen activated protein kinase or inhibit cyclin dependent entry into S-phase of non-ES cells.

40. A method according to any of Claims 1 to 8, comprising maintaining the ES cells in the presence of a compound which selectively promotes self-renewal of the ES cells.

41. A method according to any of Claim 1 to 8, comprising maintaining the ES cells in the presence of a compound which selectively inhibits propagation or survival of cells other than ES cells.

42. A method according to any of Claims 1 to 8, comprising maintaining the ES cells in the presence of a MAP kinase phosphatase.

43. A method of culture of ES cells, comprising expressing in the ES cells a compound which selectively promotes self-renewal of the ES cells and/or inhibits propagation or survival of cells other than ES cells.

44. A method according to Claim 43, comprising expressing a MAP kinase phosphatase in an ES cell.

45. Use of a compound that selectively promotes self renewal of ES cells in a method of obtaining a substantially pure culture of ES cells.

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WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 :
C12N 5/06, C12N 5/08

A3

(11) International Publication Number: WO 00/15764

(43) International Publication Date: 23 March 2000 (23.03.00)

(21) International Application Number: PCT/GB99/03031

(22) International Filing Date: 13 September 1999 (13.09.99)

(30) Priority Data:
9819912.8 11 September 1998 (11.09.98) GB

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(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(88) Date of publication of the international search report:
29 June 2000 (29.06.00)

(54) Title: PROPAGATION AND/OR DERIVATION OF EMBRYONIC STEM CELLS

(57) Abstract

Embryonic stem (ES) cells are cultured in the presence of a compound which selectively inhibits propagation or survival of cells other than ES cells. The ES cells have not been genetically altered. Instead, the compound inhibits a signalling pathway which is essential for propagation of differentiated cells but is not essential for propagation of ES cells – hence ES cells are selectively maintained in the culture.

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BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 99/03031

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N5/06 C12N5/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 30151 A (THE UNIVERSITY OF EDINBURGH) 21 August 1997 (1997-08-21)	1,2,9, 10, 12-14, 16-19, 21-23, 25,26, 28-30, 32-38, 40-43,45
Y	page 6, line 1 -page 12, paragraph 2; claims 1-10,16-32 page 22, paragraph 2	6,15,20, 24,27, 31,39

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 April 2000	27/04/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Ryckebosch, A

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	H. NIWA ET AL.: "SELF-RENEWAL OF PLURIPOTENT EMBRYONIC STEM ELLS IS MEDIATED VIA ACTIVATION OF STAT3." GENES & DEVELOPMENT , vol. 12, no. 13, 1 July 1998 (1998-07-01), pages 2048-2060, XP002135431 NEW YORK, N.Y., US page 2056, left-hand column, paragraph 3 -right-hand column, paragraph 2	6,15,20, 24,27, 31,39
A	D.T. DUDLEY ET AL.: "A SYNTHETIC INHIBITOR OF THE MITOGEN-ACTIVATED PROTEIN KINASE CASCADE." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 92, August 1995 (1995-08), pages 7686-7689, XP002135432 WASHINGTON, US cited in the application the whole document	1-45
P,X	T. BURDON ET AL.: "SUPPRESSION OF SHP-2 AND ERK SIGNALLING PROMOTES SELF-RENEWAL OF MOUSE EMBRYONIC STEM CELLS." DEVELOPMENTAL BIOLOGY, vol. 210, 1 June 1999 (1999-06-01), pages 30-43, XP000901724 NEW YORK, N.Y., US the whole document	1-45
P,X	T. BURDON ET AL.: "SIGNALING MECHANISMS REGULATING SELF-RENEWAL AND DIFFERENTIATION OF PLURIPOTENT EMBRYONIC STEM CELLS." CELLS TISSUES ORGANS, vol. 165, no. 3-4, 1999, pages 131-143, XP000891760 BASEL, CH the whole document	1-45

INTERNATIONAL SEARCH REPORT

...ion on patent family members

International Application No
PCT/GB 99/03031

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9730151 A	21-08-1997	AU 1802797 A	02-09-1997
		CA 2246712 A	21-08-1997
		EP 0880584 A	02-12-1998

PATENT COOPERATION TREATY

RECD 22 DEC 2000
WIPO PCT

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
GWS/20650		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB99/03031	13/09/1999	11/09/1998
International Patent Classification (IPC) or national classification and IPC C12N5/00		
Applicant UNIVERSITY OF EDINBURGH et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 8 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 4 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
07/04/2000	19.12.2000
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Steffen, P Telephone No. +49 89 2399 7307

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB99/03031

I. Basis of the report

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).)*:
Description, pages:

1-28 as originally filed

Claims, No.:

1-29 with telefax of 26/10/2000

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:
see separate sheet

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	14-19
	No:	Claims	1-13, 20-29
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-29
Industrial applicability (IA)	Yes:	Claims	1-29
	No:	Claims	

2. Citations and explanations
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Re Item I

Basis of the opinion

None of the figures 1-8, referred to in the description of the present application on pages 13-17, were filed with the present application. Examination is consequently carried out without the information presented in the respective figures.

The amendments filed with telefax of 26.10.2000 are in accordance with article 34(2)(b) PCT. Examination is based accordingly on the newly filed claims.

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

Reference is made to the following documents:

D1: H. NIWA ET AL.: 'SELF-RENEWAL OF PLURIPOTENT EMBRYONIC STEM ELLS IS MEDIATED VIA ACTIVATION OF STAT3.' GENES & DEVELOPMENT , vol. 12, no. 13, 1 July 1998 (1998-07-01), pages 2048-2060.

D2: WO 97 30151 A (THE UNIVERSITY OF EDINBURGH) 21 August 1997 (1997-08-21)

D3: SAVATIER ET AL.: 'WIHTDRAWAL OF DIFFERENTIATION INHIBITORY ACTIVITY/LEUKEMIA INHIBITORY FACTOR UP-REGULATES D-TYPE CYCLINS AND CYCLIN-DEPENDENT KINASE INHIBITORS IN MOUSE EMBRYONIC STEM CELLS.' ONCOGENE, vol. 12(2), 1996, pages 309-322 (abstract).

D4: D.T. DUDLEY ET AL.: 'A SYNTHETIC INHIBITOR OF THE MITOGEN-ACTIVATED PROTEIN KINASE CASCADE.' PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 92, August 1995 (1995-08), pages 7686-7689.

D5: FUKADA, T.H. ET AL.: 'TWO SIGNALS ARE NECESSARY FOR CELL PROLIFERATION INDUCED BY A CYTOKINE RECEPTOR GP130: INVOLVEMENT OF STAT3 IN ANTI-APOPTOSIS, IMMUNITY, vol.5(5), 1996, pages 449-460.

D6: WO-A-94/24274

D7: T. BURDON ET AL.: 'SUPPRESSION OF SHP-2 AND ERK SIGNALLING PROMOTES SELF-RENEWAL OF MOUSE EMBRYONIC STEM CELLS.' DEVELOPMENTAL BIOLOGY, vol. 210, 1 June 1999 (1999-06-01), pages 30-43.

D8: T. BURDON ET AL.: 'SIGNALLING MECHANISMS REGULATING SELF-RENEWAL

AND DIFFERENTIATION OF PLURIPOTENT EMBRYONIC STEM CELLS.' CELLS TISSUES ORGANS, vol. 165, no. 3-4, 1999, pages 131-143.

D5 and D6 were not cited in the international preliminary search report. A copy of the document has been appended to the written opinion. D3 is cited in the application (page 11, line 20).

Examination has been carried out, assuming a validly claimed priority right. Should the priority of the present application found not to be valid, will the documents D7 and D8 have to be considered relevant to the questions of novelty and inventive step for claims 1-45.

The present application refers to methods of culturing embryonic stem cells (ES) cells, in the presence of one or more compounds which either selectively promote self-renewal (propagation or surviving) of the ES cells and/or inhibit propagation or survival of cells other than ES cells, or both. In specific embodiments, propagation inhibition of said compounds is via a signalling pathway essential for the propagation of non-ES cells but not essential for the propagation of ES cells, involving inhibition of either cyclin dependent entry into S-phase of non-ES cells, SHP-2 enzyme, ras/MAPK cascade or MEK. Furthermore are claimed compositions, uses, culture media, methods for deriving (comprising developing an embryo, either in vitro or in vivo) or culturing ES cells all referring to the said compounds.

Claims 14-19 are not anticipated by the prior art documents on file. The corresponding subject-matter thus meets with the requirements of article 33(2) PCT.

Claims 1-13 and 20-29 lack novelty under the provisions of article 33(2) PCT for the following reasons.

The method of claims 1-6 is anticipated by D1 (D1, page 2056, left column, last paragraph, culture of ES cells in the presence of both LIF and the MEK inhibitor PD098059). Although D1 is silent towards synergistic action of both compounds as depicted in claim 1, it discloses the simultaneous use of both compounds as depicted in dependent claims 4 and 6. It is therefore inevitable to assume that in the method as described in D1, synergistic effect of both compounds LIF and PD098059 is inherent, even if this parameter was not assessed. For claims 2 and 3, it is noted here that PD098059 is inherently a specific

inhibitor of MEK and hence of reduces the activity of the ras/MAPK cascade and can therefore also be considered to reduce the activity of a mitogen activated protein kinase e.g. D4, page 7687, right column and page 7689, left column, paragraph 3, first sentence). Claim 7 is also at present assumed to be disclosed in D1, as with the information given, the skilled person would immediately recognize an acceptable range of concentration for LIF to be used. It cannot also at present not be excluded, that the disclosure of D3 (D3, abstract, culture of DIA/LIF-stimulated ES cells by overexpressing p16Ink4a, a specific inhibitor of CDK4 and CDK6) meets with all the requirements of claims 1-3 and 5-7. For evident reasons, the disclosures of D1 and D3 can also be considered novelty anticipating for claims 8-13 and claims 8, 9 and 11-13, respectively. Novelty of claims 20-24 are for similar reasons also anticipated by D1 (same citation), because for ES culture, a composition according to these claims, must inherently have been used. Referring also to the above, novelty of claims 20-22 and 24 are at present considered to be anticipated by D3 (same citations). Novelty of claim 25 is anticipated by the teachings of D1 and D3 (same citations, e.g. PD098059 and p16Ink4a and it can be assumed, that the use of the compounds, due to their biological effects, lead to the obtention of substantially pure cultures of ES cells from mixtures of differentiated cells and pure ES cells). For similar reasons as set out above, claims 26-29 are considered to be anticipated by D1 and claims 26, 27 and 29 by D3 (same citations, it also noted here that with the use of both compounds of D1 (PD098059 and DIA/LIF) and D3 (p16Ink4a and LIF), inherently substantially pure cultures of ES cells must have been obtained.

In summary, claims 1-13 and 20-29 lack novelty under the provisions of article 33(2) PCT and consequently lack also inventive activity under article 33(3) PCT.

Moreover in a more general manner, claims 1-29 lack inventive activity under the provisions of article 33(3) PCT for the following reasons.

The present application refers to methods of culturing embryonic stem cells (ES) cells, in the presence of one or more compounds which either selectively promote self-renewal (propagation or surviving) of the ES cells and/or inhibit propagation or survival of cells other than ES cells, or both. In specific embodiments, propagation inhibition of said compounds is via a signalling pathway essential for the propagation of cells other than ES cells but not essential for the propagation of ES cells, involving inhibition of either cyclin dependent entry into S-phase of non-ES cells, SHP-2 enzyme, ras/MAPK cascade or MEK.

D1 discloses that in ES cells, LIF stimulates gp130 which in turn mediates signals sufficient for ES cell renewal (D1, page 2048, right column). D1 furthermore discloses, that in the context of gp130, by inhibition of MEK, that the MAPK/ERK signalling pathway activation is not involved in the stem cell colony formation in responses to LIF, neither is SHP-2 in the context of the Ras-ERK cascade involved in self-renewal of ES cells (D1, page 2056, left column, last paragraph). D3 discloses that inhibition of CDK4 by p16Ink4a e.g. inhibition of cyclin-dependent entry into S-phase does not arrest growth of LIF-stimulated ES cells (D3, abstract). It is also known in the art, that for cells other than ES cells, the ERK cascade and in the context of gp130, SHP-2 and the activation of MAP kinase are mediators of cell propagation e.g. see for example D4 and D5. In summary the prior art taken together discloses that propagation of non-ES cells is linked in the gp130 context to SHP-2, MAPK/ERK-MEK, whereas this is not the case in ES cells stimulated with LIF (e.g. a compound that selectively promotes self-renewal of ES cells). Furthermore, a constant aim in the ES cell culture is to produce cell populations of a satisfactorily low degree of heterogeneity and to reduce the presence of non-pluripotent, differentiated cell types from the cultures (see D6, page 2, second paragraph). The instantly proposed solution of this problem, as set forth in claims 1-3 and 5 and the more specific solutions as referred to in claims 4, 6 and 7, was obvious to the skilled person when considering the prior art disclosures as mentioned above, which either suggest and employ the claimed methods (claims 1-7) or suggest their obviousness (in the case of SHP-2, claim 3). It is noted here the mere observation of an additional parameter for the method of claim 1 (e.g. "synergistic") cannot be taken in account for inventive step since it is only the result of defined method steps that are carried out, said method steps being obvious over the prior art as mentioned above. All further claims, referring directly or indirectly to the said methods and compounds of claims 1-7, constitute obvious subject- matter in light of claims 1-7 and are considered therefore also as obvious for the skilled person.

In consequence, claims 1-29 lack inventive activity under the provisions of article 33(3) PCT.

Re Item VIII

Certain observations on the international application

The following observations concern article 6 PCT (clarity).

Claims 1, 2 and 8 lack clarity in that characterising technical features which define how the methods have to be carried out are missing e.g. what is the compound that promotes self-renewal and propagation inhibition, what is the cell signalling pathway to be inhibited? Furthermore it is unclear how the parameter "synergistic" is to be construed e.g. when and in which test conditions is the combination of compounds (a) and (b) understood to be synergistic.

Referring to this, claim 26 also lacks characterising technical features in respect to "a compound that enhances the response of ES cells". Here it is also not clear when a "compound" is considered to enhance such "response" and how this is assessed.

Claims 7 and 13 refer to LIF concentrations of "about 5 U/ml". First is the term "about" unclear because subjectively interpretable. Second is a definition for the units U missing, as methods of assessing units can vary from laboratory to laboratory and from supplier to supplier.

In a similar manner, through the use of the undefined term "a compound", claims 1, 2, 8, 14, 15, 17, 18, 20, 21, 25 and 26 (also the term "an agent"), lack characterising technical features and are therefore unclear.

Claim 20 is unclear since it cannot readily be appreciated what is understood with a "synergistic amount".

The term "substantially" in claim 25 is prone to subjective interpretation and therefore unclear.

CLAIMS

1. A method of culture of embryonic stem (ES) cells comprising maintaining a culture of ES cells in the presence of:-

(a) a compound that promotes propagation or survival of ES cells; and

(b) a compound that inhibits propagation or survival of cells other than ES cells;

wherein the combination of compounds (a) and (b) is synergistic.

2. A method according to Claim 1 wherein compound (b) selectively inhibits a signalling pathway essential to propagation or survival of cells other than ES cells.

3. A method according to any of Claims 1 or 2, wherein compound (b) is selected from compounds which inhibit activity of the enzyme SHP-2, inhibit the ras/MAPK cascade, inhibit MEK, inhibit a mitogen activated protein kinase or inhibit cyclin dependent entry into S-phase of non-ES cells.

4. A method according to Claim 3, wherein compound (b) is PD098059.

5. A method according to any previous Claims, wherein compound (a) is a cytokine that activates the cytokine receptor gp130 in ES cells.

6. A method according to Claim 5, wherein the cytokine is Leukaemia Inhibitory Factor (LIF).

7. A method according to Claim 6, wherein the concentration of LIF is about 5 U/ml.

8. A method of selecting ES cells from a mixed culture of cells comprising

exposing the mixed culture of cells to:-

(a) a compound that promotes propagation or survival of ES cells; and

(b) a compound that inhibits propagation or survival of cells other than ES cells;

wherein the combination of compounds (a) and (b) is synergistic.

9. A method according to Claims 8, wherein compound (b) is selected from compounds which inhibit activity of the enzyme SHP-2, inhibit the ras/MAPK cascade, inhibit MEK, inhibit a mitogen activated protein kinase or inhibit cyclin dependent entry into S-phase of non-ES cells.

10. A method according to Claim 9, wherein compound (b) is PD098059.

11. A method according to any of Claims 8 to 10, wherein compound (a) is a cytokine that activates the cytokine receptor gp130 in ES cells.

12. A method according to Claim 11, wherein the cytokine is Leukaemia Inhibitory Factor (LIF).

13. A method according to Claim 12, wherein the concentration of LIF is about 5 U/ml.

14. A method of deriving ES cells comprising isolating cells from a non-human embryo or embryoid body and maintaining a culture of those cells in the presence of a compound that selectively inhibits propagation or survival of cells other than ES cells.

15. A method according to Claim 14 comprising dissociating cells obtained and then further maintaining the dissociated cells in the presence of the or a further

compound that selectively inhibits propagation or survival of cells other than ES cells.

16. A method according to Claims 14 or 15 comprising developing an embryo *in vivo*, harvesting the embryo prior to pro-amniotic cavity formation and isolating cells therefrom.

17. A method of deriving ES cells comprising developing a non-human embryo *in vitro*, isolating cells from the inner cell mass of the embryo and maintaining those cells in the presence of a compound that selectively inhibits propagation or survival of cells other than ES cells.

18. A method according to Claim 17 comprising removing primitive endoderm prior to culture in the presence of the compound.

19. A method according to any of Claims 14 to 18, wherein the compound that selectively inhibits propagation or survival of cells other than ES cells is selected from compounds which inhibit activity of the enzyme SHP-2, inhibit the ras/MAPK cascade, inhibit MEK, inhibit a mitogen activated protein kinase or inhibit cyclin dependent entry into S-phase of non-ES cells.

20. A composition for selective culture of ES cells, comprising (a) a compound that inhibits differentiation of ES cells, and (b) a synergistic amount of a compound that inhibits propagation or survival of cells other than ES cells.

21. A composition according to Claim 20 wherein compound (b) selectively inhibits a signalling pathway essential to propagation or survival of cells other than ES cells.

22. A composition according to Claim 20 or 21, wherein compound (b) is selected from compounds which inhibit activity of the enzyme SHP-2, inhibit the ras/MAPK cascade, inhibit MEK, inhibit a mitogen activated protein kinase or inhibit

cyclin dependent entry into S-phase of non-ES cells.

23. A composition according to Claim 22, wherein compound (b) is PD098059.

24. A composition according to any of Claims 20 to 23 wherein compounds (a) is a cytokine that binds to the cytokine receptor gp130, such as LIF.

25. Use of a compound that selectively inhibits propagation or survival of cells other than ES cells in a method of obtaining a substantially pure culture of ES cells from a mixed cell culture.

26. Use of a compound that enhances the response of ES cells to an agent that promotes propagation or survival of ES cells, in a method for a obtaining a substantially pure culture of ES cells.

27. Use according to claim 26, wherein the compound is selected from compounds which inhibit activity of the enzyme SHP-2, inhibit the ras/MAPK cascade, inhibit MEK, inhibit a mitogen activated protein kinase or inhibit cyclin dependent entry into S-phase of non-ES cells.

28. Use according to Claim 27, wherein the compound is PD098059.

29. Use according to Claims 26 to 28, wherein the agent is LIF.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
GWS/20650		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/GB 99/ 03031	13/09/1999	11/09/1998
Applicant		
UNIVERSITY OF EDINBURGH et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. --

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [ ] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 99/03031

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N5/06 C12N5/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 30151 A (THE UNIVERSITY OF EDINBURGH) 21 August 1997 (1997-08-21)	1,2,9, 10, 12-14, 16-19, 21-23, 25,26, 28-30, 32-38, 40-43,45
Y	page 6, line 1 -page 12, paragraph 2; claims 1-10,16-32 page 22, paragraph 2	6,15,20, 24,27, 31,39

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 April 2000	27/04/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Ryckebosch, A

2

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	H. NIWA ET AL.: "SELF-RENEWAL OF PLURIPOTENT EMBRYONIC STEM ELLS IS MEDIATED VIA ACTIVATION OF STAT3." GENES & DEVELOPMENT , vol. 12, no. 13, 1 July 1998 (1998-07-01), pages 2048-2060, XP002135431 NEW YORK, N.Y., US page 2056, left-hand column, paragraph 3 -right-hand column, paragraph 2 ---	6,15,20, 24,27, 31,39
A	D.T. DUDLEY ET AL.: "A SYNTHETIC INHIBITOR OF THE MITOGEN-ACTIVATED PROTEIN KINASE CASCADE." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 92, August 1995 (1995-08), pages 7686-7689, XP002135432 WASHINGTON, US cited in the application the whole document ---	1-45
P,X	T. BURDON ET AL.: "SUPPRESSION OF SHP-2 AND ERK SIGNALLING PROMOTES SELF-RENEWAL OF MOUSE EMBRYONIC STEM CELLS." DEVELOPMENTAL BIOLOGY, vol. 210, 1 June 1999 (1999-06-01), pages 30-43, XP000901724 NEW YORK, N.Y., US the whole document ---	1-45
P,X	T. BURDON ET AL.: "SIGNALING MECHANISMS REGULATING SELF-RENEWAL AND DIFFERENTIATION OF PLURIPOTENT EMBRYONIC STEM CELLS." CELLS TISSUES ORGANS, vol. 165, no. 3-4, 1999, pages 131-143, XP000891760 BASEL, CH the whole document -----	1-45

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
T/GB 99/03031

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9730151 A	21-08-1997	AU 1802797 A	02-09-1997
		CA 2246712 A	21-08-1997
		EP 0880584 A	02-12-1998

Our Ref: GWS/DJC/20650
Yr. Ref: -

FOR THE ATTENTION OF THE INTERNATIONAL PRELIMINARY EXAMINATION AUTHORITY

European Patent Office
Directorate General 2
D-80298 Munich
Germany

27 October 2000

Dear Sirs

International Patent Application No. PCT/GB99/03031
University of Edinburgh

We refer to the Written Opinion issued by the International Examiner on 27 June 2000, the deadline for reply to which has been extended by one month as confirmed in your Communication of 4 October 2000.

We enclose amended claims pages 1-4 (in triplicate) and previous claims pages 29-34 with manuscript amendments (one copy). In the facsimile version of this letter we enclose one copy of the amended claims pages 1-4.

Amendments

Claims 1-11, 23-33 and 40-45 have been deleted. New Claims 4, 7-13, 23 and 26-29 have been inserted. The remaining claims have been renumbered and their dependencies amended accordingly.

The amendments to the remaining claims are described in more detail below.

Previous Claim 12 has been combined with previous Claim 13 to form new Claim 1.

Previously dependant Claims 14 and 15 are renumbered Claim 2 and 3 and a new Claim 4 has been inserted. New Claim 4 recites a method according to Claim 3 wherein compound (b) is PD098059.

Continued ...

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Basis for the new Claim 4 resides in the specification and in particular on pages 23 and 24.

New Claims 10, 23 and 28 correspond with new Claim 4.

A new Claim 7 has been inserted that recites that the concentration of LIF is about 5U/ml. Basis for this new claim lies in the specification on page 23 (line 9).

A corresponding new claim, Claim 13, has also been inserted.

Previous Claims 34-39 have been renumbered as Claims 14-19.

New Claims 14 and 17 (corresponding to previous Claims 34 and 37) have been amended by insertion of the phrase "a non-human" prior to the first recitation of the word "embryo" in both claims. The amendment is in accordance with the Examiner's direction in order to exclude the development of human embryos from the subject matter of the present invention.

Novelty

The Examiner has objected to the claims of the present invention on the grounds that they lack novelty with regard to the prior art of D1-D4.

D1 states that the MEK inhibitor PD098059 specifically blocks activation of the ERK kinases but does not inhibit stem cell colony formation in response to LIF (page 2056, column 1, last paragraph).

D2 mentions that ES cells cultured in the presence of DIA/LIF or of a feeder layer of DIA/LIF-producing cells maintain their proliferative capacity, retain characteristic stem cell morphology, and express stem cell markers (page 2, bottom paragraph).

D3 is the abstract by the paper by Savatier *et al* and mentions that DIA/LIF-stimulated ES cells are not growth-arrested by over expression of a specific inhibitor of CDK4 and CDK6. D3 is mentioned in the present specification on page 11 (line 20).

D4 relates to the identification and characterisation of the MAPK/ERK kinase (MEK) inhibiting compound PD098059.

Claim 1 has been amended to recite a method of culture of ES cells in the presence of (a) a compound that promotes propagation or survival of ES cells, and (b) a compound that inhibits propagation or survival of cells other than ES cells, wherein the combination of these separate compounds is synergistic. Thus, Claim 1 clearly recites a method whereby the compounds (a) and (b) work together in a synergistic fashion rather than merely co-exist in combination.

D1-D4 do not describe a method of culturing ES cells comprising maintaining the ES cells in the presence of a synergistic combination of compounds.

D1 and D3 describe the culture of ES cells in the presence of DIA/LIF together with an inhibitor of an intra-cellular signalling pathway. There is no mention that the inhibitor has a synergistic relationship with the DIA/LIF. All that is stated for both D1 and D3 is that the inhibitor does not prevent DIA/LIF-stimulated ES cell regeneration and renewal. Claim 1 as amended is therefore novel over D1 and D3.

Continued ...

D2 only refers to the maintenance of ES cell culture in the presence of DIA/LIF and does not describe a method of culture with further compound that acts in a synergistic manner with the compound that promotes propagation or survival of ES cells (in this case DIA/LIF). Claim 1 as amended is therefore also novel over D2.

New Claim 8 recites a method of selecting ES cells from a mixed culture of cells comprising exposing the mixed culture of cells to a synergistic combination of compounds (a) and (b), wherein compound (a) promotes propagation or survival of ES cells and (b) inhibits propagation or survival of cells other than ES cells. None of the prior art documents, especially D1-D3, recite a method for selecting ES cells from a mixed culture. Also, for the same reasons as those given for Claim 1 none of the cited prior art documents describe synergistic combinations that facilitate the selection of ES cells and therefore Claim 8 is novel.

The Examiner has objected to previous Claims 18-21, renumbered as Claims 20-24 (incorporating new Claim 23), on the grounds that they are anticipated by D1.

Claim 20 recites a composition comprising a compound that inhibits differentiation of ES cells together with a synergistic amount of a compound that inhibits propagation or survival of cells other than ES cells. As mentioned previously, D1 does not describe a synergistic combination of factors. D1 merely states that inhibition of MEK does not prevent DIA/LIF induced colony formation in ES cells. Since there is no recitation of synergy in D1 it does not anticipate the subject matter of Claim 20-24.

Claim 25 (corresponding to previous Claim 22) has been objected to on the grounds that it is anticipated by D1 and D3. Claim 25 has been amended by insertion of the proviso that the use of the compound is applied to obtaining a substantially pure culture of ES cells from a mixed cell culture. D1 and D3 both describe the effect of a cell signalling inhibitor on the ability of ES cells to grow and renew. Neither D1 nor D3 describe the use of a compound for purifying ES cells from a mixed cell culture. Thus Claim 25 as amended is novel with respect to D1 and D3.

New Claim 26 recites a use of a compound that enhances the response of ES cells to an agent that promotes propagation or survival of ES cells in a method for obtaining a substantially pure culture of ES cells. The use of a compound to enhance response to a second compound (the agent) is not disclosed in the cited prior art. Neither D1 nor D3 disclose any form of interaction between the inhibitor compound and DIA/LIF. Claim 26 and dependent Claims 27-29 are therefore novel over the prior art.

Inventive Step

Problem:
The present invention seeks to address the problem of obtaining and stably culturing ES cells, and in particular to reduce the rate of differentiation of ES cells in known cultures.

Solution:
The invention provides for maintenance of ES cells in the presence of compounds that promote propagation or survival of ES cells and compounds that inhibit propagation or survival of cells other than ES cells wherein the combination of these compounds is synergistic - i.e. the effects of combining these compounds is supra-additive.

Continued ...

One such combination of synergistic compounds is described in the present specification in the paragraphs bridging pages 23 and 24 (lines 31-32 on page 23 to lines 1-8 on page 24). In this example, a compound that promotes propagation or survival of ES cells is DIA/LIF, and the compound that inhibits survival of cells other than ES cells is the MEK inhibitor PD098059. The surprising result obtained when these two compounds were combined in cell culture medium was that ES cell response DIA/LIF was enhanced and thus the level of self-renewal increased (page 23, line 14). Thus, the effect on ES cells observed in the presence of these two compounds was more than mere combination and demonstrated a synergy or supra-additive effect.

The Examiner has identified D1 as the closest prior art. D1 only mentions that inhibition of the MAPK/ERK signalling pathway by PD098059 does not inhibit growth of ES cell colonies in the presence of DIA/LIF. There is no discussion in D1 of the synergistic effect observed nor the enhancement of response to DIA/LIF in ES cells that is described in the present application. Thus, D1 does not teach that such a synergistic effect exists and it would not have been obvious to a person of skill in the art to combine such compounds in order to obtain a synergistic effect that is beneficial to the culturing of ES cells.

The Examiner considers that the properties of compounds such as PD098059 that inhibit the MAPK/ERK signalling pathway together with a compound that promotes propagation of ES cells would have been an obvious development. Similarly, the Examiner is of the opinion that D3, which describes an alternative intra-cellular signalling inhibitor in combination with DIA/LIF stimulated ES cells, would also teach the methods and compositions of the present invention.

When assessing prior art such as D1 and D3 it is essential to recognise that both of these documents briefly describe experiments in which the only reported effect is that growth and renewal of ES cells cultured in DIA/LIF is not inhibited in the presence of specific inhibitors of intra-cellular signalling pathways. There is no mention of analysis performed on the general quality or health of the ES cells in culture, nor of their suitability for long term growth, regeneration and subsequent differentiation.

A person of skill in the art would recognise that the specific inhibitors mentioned in D1 and D3, namely PD098059 and p16Ink4a are normally regarded a potent inhibitors of cell growth (for example see document D4 with regards to PD098059 inhibition of cell growth). It would take a significant step to go from the reported observations in D1 and D3 that such potent inhibitors do not block DIA/LIF induced growth, to arriving at the conclusion that they are suitable for inclusion in ES cell culture media. The present description clearly states that PD098059 impairs the growth of ES cells at concentrations greater than 50 micromolar (page 23 lines 15-18). It would not be obvious, therefore, to utilise such inhibitors in ES cell culture media, nor would it be obvious that combination of such potent inhibitors with a ES cell propagation promoting compound such as DIA/LIF would give a synergistic and enhanced response.

The amended claims therefore demonstrate an inventive step over the cited prior art.

The applicants wish to defer addressing the Examiner's clarity objections until entry into the national and regional phases.

Continued ...

We look forward to issue of the IPER.

Yours faithfully

George W Schlich
MATHYS & SQUIRE

Enc: Amended claims pages 1-3 (in triplicate).
Claims pages 29-34 with manuscript amendments (one copy only).

/cm

CLAIMS

1. A method of culture of embryonic stem (ES) cells comprising maintaining a culture of ES cells in the presence of:-

(a) a compound that promotes propagation or survival of ES cells; and

(b) a compound that inhibits propagation or survival of cells other than ES cells;

wherein the combination of compounds (a) and (b) is synergistic.

2. A method according to Claim 1 wherein compound (b) selectively inhibits a signalling pathway essential to propagation or survival of cells other than ES cells.

3. A method according to any of Claims 1 or 2, wherein compound (b) is selected from compounds which inhibit activity of the enzyme SHP-2, inhibit the ras/MAPK cascade, inhibit MEK, inhibit a mitogen activated protein kinase or inhibit cyclin dependent entry into S-phase of non-ES cells.

4. A method according to Claim 3, wherein compound (b) is PD098059.

5. A method according to any previous Claims, wherein compound (a) is a cytokine that activates the cytokine receptor gp130 in ES cells.

6. A method according to Claim 5, wherein the cytokine is Leukaemia Inhibitory Factor (LIF).

7. A method according to Claim 6, wherein the concentration of LIF is about 5 U/ml.

8. A method of selecting ES cells from a mixed culture of cells comprising

exposing the mixed culture of cells to:-

(a) a compound that promotes propagation or survival of ES cells; and

(b) a compound that inhibits propagation or survival of cells other than ES cells;

wherein the combination of compounds (a) and (b) is synergistic.

9. A method according to Claims 8, wherein compound (b) is selected from compounds which inhibit activity of the enzyme SHP-2, inhibit the ras/MAPK cascade, inhibit MEK, inhibit a mitogen activated protein kinase or inhibit cyclin dependent entry into S-phase of non-ES cells.

10. A method according to Claim 9, wherein compound (b) is PD098059.

11. A method according to any of Claims 8 to 10, wherein compound (a) is a cytokine that activates the cytokine receptor gp130 in ES cells.

12. A method according to Claim 11, wherein the cytokine is Leukaemia Inhibitory Factor (LIF).

13. A method according to Claim 12, wherein the concentration of LIF is about 5 U/ml.

14. A method of deriving ES cells comprising isolating cells from a non-human embryo or embryoid body and maintaining a culture of those cells in the presence of a compound that selectively inhibits propagation or survival of cells other than ES cells.

15. A method according to Claim 14 comprising dissociating cells obtained and then further maintaining the dissociated cells in the presence of the or a further

compound that selectively inhibits propagation or survival of cells other than ES cells.

16. A method according to Claims 14 or 15 comprising developing an embryo *in vivo*, harvesting the embryo prior to pro-amniotic cavity formation and isolating cells therefrom.

17. A method of deriving ES cells comprising developing a non-human embryo *in vitro*, isolating cells from the inner cell mass of the embryo and maintaining those cells in the presence of a compound that selectively inhibits propagation or survival of cells other than ES cells.

18. A method according to Claim 17 comprising removing primitive endoderm prior to culture in the presence of the compound.

19. A method according to any of Claims 14 to 18, wherein the compound that selectively inhibits propagation or survival of cells other than ES cells is selected from compounds which inhibit activity of the enzyme SHP-2, inhibit the ras/MAPK cascade, inhibit MEK, inhibit a mitogen activated protein kinase or inhibit cyclin dependent entry into S-phase of non-ES cells.

20. A composition for selective culture of ES cells, comprising (a) a compound that inhibits differentiation of ES cells, and (b) a synergistic amount of a compound that inhibits propagation or survival of cells other than ES cells.

21. A composition according to Claim 20 wherein compound (b) selectively inhibits a signalling pathway essential to propagation or survival of cells other than ES cells.

22. A composition according to Claim 20 or 21, wherein compound (b) is selected from compounds which inhibit activity of the enzyme SHP-2, inhibit the ras/MAPK cascade, inhibit MEK, inhibit a mitogen activated protein kinase or inhibit

cyclin dependent entry into S-phase of non-ES cells.

23. A composition according to Claim 22, wherein compound (b) is PD098059.

24. A composition according to any of Claims 20 to 23 wherein compounds (a) is a cytokine that binds to the cytokine receptor gp130, such as LIF.

25. Use of a compound that selectively inhibits propagation or survival of cells other than ES cells in a method of obtaining a substantially pure culture of ES cells from a mixed cell culture.

26. Use of a compound that enhances the response of ES cells to an agent that promotes propagation or survival of ES cells, in a method for a obtaining a substantially pure culture of ES cells.

27. Use according to claim 26, wherein the compound is selected from compounds which inhibit activity of the enzyme SHP-2, inhibit the ras/MAPK cascade, inhibit MEK, inhibit a mitogen activated protein kinase or inhibit cyclin dependent entry into S-phase of non-ES cells.

28. Use according to Claim 27, wherein the compound is PD098059.

29. Use according to Claims 26 to 28, wherein the agent is LIF.

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
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Date of mailing (day/month/year) 04 May 2000 (04.05.00)	
International application No. PCT/GB99/03031	**Applicant's or agent's file reference** GWS/20650
International filing date (day/month/year) 13 September 1999 (13.09.99)	**Priority date** (day/month/year) 11 September 1998 (11.09.98)
Applicant SMITH, Austin, Gerard et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

07 April 2000 (07.04.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

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PCT

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only

International Application No.

International Filing Date

Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference *(if desired) (12 characters maximum)* GWS/20650

Box No. I TITLE OF INVENTION

PROPAGATION AND/OR DERIVATION OF EMBRYONIC STEM CELLS

Box No. II APPLICANT

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

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State *(that is, country)* of nationality: GB

State *(that is, country)* of residence: GB

This person is applicant for the purposes of: ☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

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This person is:

☐ applicant only

☒ applicant and inventor

☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is,country)* of nationality: GB

State *(that is, country)* of residence: GB

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

☒ Further applicants and/or (further) inventors are indicated on a continuation sheet.

Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☒ agent ☐ common representative

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See Notes to the request form

Continuation of Box No. III FURTHER APPLICANTS AND/OR (FURTHER) INVENTORS

If none of the following sub-boxes is used, this sheet should not be included in the request.

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This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: GB

State *(that is, country)* of residence: GB

This person is applicant for the purposes of:
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This person is:
- ☐ applicant only
- ☐ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality:

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This person is applicant for the purposes of:
- ☐ all designated States
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This person is:
- ☐ applicant only
- ☐ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

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This person is:
- ☐ applicant only
- ☐ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purposes of:
- ☐ all designated States
- ☐ all designated States except the United States of America
- ☐ the United States of America only
- ☐ the States indicated in the Supplemental Box

☐ Further applicants and/or (further) inventors are indicated on another continuation sheet.

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- [x] KZ Kazakhstan
- [x] LC Saint Lucia
- [x] LK Sri Lanka
- [x] LR Liberia
- [x] LS Lesotho
- [x] LT Lithuania
- [x] LU Luxembourg
- [x] LV Latvia
- [x] MD Republic of Moldova
- [x] MG Madagascar
- [x] MK The former Yugoslav Republic of Macedonia
- [x] MN Mongolia
- [x] MW Malawi
- [x] MX Mexico
- [x] NO Norway
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- [x] SK Slovakia
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- [x] TM Turkmenistan
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- [x] ALL OTHER STATES PARTY TO THE PCT

Precautionary Designation Statement: In addition to the designations made above, the applicant also makes under Rule 4.9(b) all other designations which would be permitted under the PCT except any designation(s) indicated in the Supplemental Box as being excluded from the scope of this statement. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. *(Confirmation of a designation consists of the filing of a notice specifying that designation and the payment of the designation and confirmation fees. Confirmation must reach the receiving Office within the 15-month time limit.)*

See Notes to the request form

Supplemental Box *If the Supplemental Box is not used, this sheet should not be included in the request.*

*1. If, in any of the Boxes, **the space is insufficient** to furnish all the information: in such case, write "Continuation of Box No. ..." [indicate the number of the Box] and furnish the information in the same manner as required according to the captions of the Box in which the space was insufficient, in particular:*

*(i) **if more than two persons are involved as applicants and/or inventors** and no "continuation sheet" is available: in such case, write "Continuation of Box No. III" and indicate for each additional person the same type of information as required in Box No. III. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below;*

*(ii) if, in Box No. II or in any of the sub-boxes of Box No. III, the indication **"the States indicated in the Supplemental Box"** is checked: in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the applicant(s) involved and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is applicant;*

*(iii) if, in Box No. II or in any of the sub-boxes of Box No. III, **the inventor or the inventor/applicant is not inventor for the purposes of all designated States or for the purposes of the United States of America**: in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the inventor(s) and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is inventor;*

*(iv) if, in addition to the agent(s) indicated in Box No. IV, there are **further agents**: in such case, write "Continuation of Box No. IV" and indicate for each further agent the same type of information as required in Box No. IV;*

*(v) if, in Box No. V, the name of any State (or OAPI) is accompanied by the indication **"patent of addition,"** or **"certificate of addition,"** or if, in Box No. V, the name of the United States of America is accompanied by an indication **"continuation"** or **"continuation-in-part"**: in such case, write "Continuation of Box No. V" and the name of each State involved (or OAPI), and after the name of each such State (or OAPI), the number of the parent title or parent application and the date of grant of the parent title or filing of the parent application;*

*(vi) if, in Box No. VI, there are **more than three earlier applications whose priority is claimed**: in such case, write "Continuation of Box No. VI" and indicate for each additional earlier application the same type of information as required in Box No. VI;*

*(vii) if, in Box No. VI, **the earlier application is an ARIPO application**: in such case, write "Continuation of Box No. VI", specify the number of the item corresponding to that earlier application and indicate at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed.*

*2. If, with regard to the **precautionary designation statement** contained in Box No. V, the applicant wishes to exclude any State(s) from the scope of that statement: in such case, write "Designation(s) excluded from precautionary designation statement" and indicate the name or two-letter code of each State so excluded.*

*3. If the applicant claims, in respect of any designated Office, the benefits of provisions of the national law concerning **non-prejudicial disclosures or exceptions to lack of novelty**: in such case, write "Statement concerning non-prejudicial disclosures or exceptions to lack of novelty" and furnish that statement below.*

Continuation of Box IV

ADDITIONAL REPRESENTATIVES

RITTER; Stephen David
GARRATT; Peter Douglas
MOIR; Michael Christopher
COZENS; Paul Dennis
KAZI; Ilya
COLMER; Stephen Gary
INGRAM; Brian Victor
SIMONS; Elisabeth Anne

ALL AT:

Mathys & Squire
100 Gray's Inn Road
London
WC1X 8AL

Box No. VI PRIORITY CLAIM ☐ Further priority claims are indicated in the Supplemental Box.

Filing date of earlier application (day/month/year)	Number of earlier application	Where earlier application is: national application: country	regional application:* regional Office	international application: receiving Office
item (1) 11.09.98 GB	9819912.8	GB		
item (2)				
item (3)				

☐ The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) *(only if the earlier application was filed with the Office which for the purposes of the present international application is the receiving Office)* identified above as item(s): (1)

* *Where the earlier application is an ARIPO application, it is mandatory to indicate in the Supplemental Box at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed (Rule 4.10(b)(ii)). See Supplemental Box.*

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) *(if two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used):*

ISA /

Request to use results of earlier search; reference to that search *(if an earlier search has been carried out by or requested from the International Searching Authority):*

Date *(day/month/year)* Number Country *(or regional Office)*

Box No. VIII CHECK LIST; LANGUAGE OF FILING

This international application contains the following **number of sheets:**

request	: 5
description (excluding sequence listing part)	: 28
claims	: 6
abstract	: 1
drawings	: -
sequence listing part of description	: -
Total number of sheets	: 40

This international application is **accompanied by** the item(s) marked below:

1. ☐ fee calculation sheet
2. ☐ separate signed power of attorney
3. ☐ copy of general power of attorney; reference number, if any:
4. ☐ statement explaining lack of signature
5. ☐ priority document(s) identified in Box No. VI as item(s):
6. ☐ translation of international application into (language):
7. ☐ separate indications concerning deposited microorganism or other biological material
8. ☐ nucleotide and/or amino acid sequence listing in computer readable form
9. ☒ other *(specify)*: FORM 23/77

Figure of the drawings which should accompany the abstract:

Language of filing of the international application: ENGLISH

Box No. IX SIGNATURE OF APPLICANT OR AGENT

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).

SCHLICH; GEORGE WILLIAM
13 September 1999

For receiving Office use only

1. Date of actual receipt of the purported international application:	2. Drawings: ☐ received: ☐ not received:
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:	
4. Date of timely receipt of the required corrections under PCT Article 11(2):	
5. International Searching Authority (if two or more are competent): ISA /	6. ☐ Transmittal of search copy delayed until search fee is paid.

For International Bureau use only

Date of receipt of the record copy by the International Bureau:

Patents Form 23/77

Patents Act 1977
Rules 6, 52, 119

The Patent Office

23/77

Request for a certificate of the Comptroller or a certified or uncertified copy from a file or the register

(See the notes on the back of this form)

The Patent Office

Cardiff Road
Newport
Gwent NP9 1RH

1. Your reference

GWS/20650

2. Patent application or patent number(s)
(see notes (c) & (d))

GB 9819912.8

3. Full name of the or of each patent applicant or proprietor
(if known)

University of Edinburgh

4. What do you want a copy of? *(see note (f))*

(ii)

5. How many copies do you need?

ONE

6. State the type of certificate you want *(see note (g))* and if it is needed to support applications made outside the United Kingdom, list the countries concerned *(see notes (j) & (k))*

certified with signature and seal for filing in an International application

7. Name, address and postcode of the or of each person making this request
(see note (h))

Mathys & Squire
100 Grays Inn Road
London, WC1X 8AL

8. Name, address and postcode of the or of each person certificates or copies should be sent to (if different from that given in part 6 above)
(see note (i))

9. Signature

Mathys Squire

MATHYS & SQUIRE

Date

13 September 1999

10. Name and daytime telephone number of person to contact in the United Kingdom

George W Schlich 0171 830 0000

The demand must be filed directly with the competent International Preliminary Examining Authority or, if two or more Authorities are competent, with the one chosen by the applicant. The full name or two-letter code of that Authority may be indicated by the applicant on the line below:

IPEA/ _____

PCT

CHAPTER II

DEMAND

under Article 31 of the Patent Cooperation Treaty:
The undersigned requests that the international application specified below be the subject of international preliminary examination according to the Patent Cooperation Treaty and hereby elects all eligible States (except where otherwise indicated).

For International Preliminary Examining Authority use only

Identification of IPEA	Date of receipt of DEMAND

Box No. I IDENTIFICATION OF THE INTERNATIONAL APPLICATION

Applicant's or agent's file reference: GWS/20650

International application No.	International filing date *(day/month/year)*	(Earliest) Priority date *(day/month/year)*
PCT/GB99/03031	13 SEPTEMBER 1999	11 SEPTEMBER 1998

Title of invention

PROPOGATION AND/OR DERIVATION OF EMBROYNIC STEM CELLS

Box No. II APPLICANT(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

UNIVERISTY OF EDINBURGH
OLD COLLEGE
SOUTH BRIDGE
EDINBURGH
EH8 9YL, GB

Telephone No.:

Facsimile No.:

Teleprinter No.:

State *(that is, country)* of nationality:	State *(that is, country)* of residence:
GB	GB

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

SMITH; Austin Gerard
c/o UNIVERSITY OF EDINBURGH
CENTRE FOR GENOME RESEARCH
THE KING'S BUILDINGS
WEST MAINS ROAD
EDINBURGH, EH9 3JQ, GB

State *(that is, country)* of nationality:	State *(that is, country)* of residence:
GB	GB

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

BURDON; Thomas Grant
c/o UNIVERSITY OF EDINBURGH
CENTRE FOR GENOME RESEARCH
THE KING'S BUILDINGS
WEST MAINS ROAD
EDINBURGH, EH9 3JQ, GB

State *(that is, country)* of nationality:	State *(that is, country)* of residence:
GB	GB

☐ Further applicants are indicated on a continuation sheet.

International application No.
PCT/GB99/03031

Box No. III AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The following person is [x] agent [ ] common representative

and [x] has been appointed earlier and represents the applicant(s) also for international preliminary examination.

[ ] is hereby appointed and any earlier appointment of (an) agent(s)/common representative is hereby revoked.

[ ] is hereby appointed, specifically for the procedure before the International Preliminary Examining Authority, in addition to the agent(s)/common representative appointed earlier.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

SCHLICH; George William
MATHYS & SQUIRE
100 GRAY'S INN ROAD
LONDON
WC1X 8AL

Telephone No.: 020 7830 0000

Facsimile No.: 020 7830 0001

Teleprinter No.: -

[ ] **Address for correspondence:** Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special addr ess to which correspondence should be sent.

Box No. IV BASIS FOR INTERNATIONAL PRELIMINARY EXAMINATION

Statement concerning amendments:*

1. The applicant wishes the international preliminary examination to start on the basis of:

[ ] the international application as originally filed

the description [ ] as originally filed
[ ] as amended under Article 34

the claims [ ] as originally filed
[ ] as amended under Article 19 (together with any accompanying statement)
[ ] as amended under Article 34

the drawings [ ] as originally filed
[ ] as amended under Article 34

2. [ ] The applicant wishes any amendment to the claims under Article 19 to be considered as reversed.

3. [ ] The applicant wishes the start of the international preliminary examination to be postponed until the expiration of 20 months from the priority date unless the International Preliminary Examining Authority receives a copy of any amendments made under Article 19 or a notice from the applicant that he does not wish to make such amendments (Rule 69.1(d)). *(This check-box may be marked only where the time limit under Article 19 has not yet expired.)*

* Where no check-box is marked, international preliminary examination will start on the basis of the international application as originally filed or, where a copy of amendments to the claims under Article 19 and/or amendments of the international application under Article 34 are received by the International Preliminary Examining Authority before it has begun to draw up a written opinion or the international preliminary examination report, as so amended.

Language for the purposes of international preliminary examination: ENGLISH

[x] which is the language in which the international application was filed.
[ ] which is the language of a translation furnished for the purposes of international search.
[ ] which is the language of publication of the international application.
[ ] which is the language of the translation (to be) furnished for the purposes of international preliminary examination.

Box No. V ELECTION OF STATES

The applicant hereby elects all eligible States *(that is, all States which have been designated and which are bound by Chapter II of the PCT)*

excluding the following States which the applicant wishes not to elect:

International application No.
PCT/GB99/03031

Box No. VI CHECK LIST

The demand is accompanied by the following elements, in the language referred to in Box No. IV, for the purposes of international preliminary examination:			For International Preliminary Examining Authority use only: received	not received
1. translation of international application	:	sheets	☐	☐
2. amendments under Article 34	:	sheets	☐	☐
3. copy (or, where required, translation) of amendments under Article 19	:	sheets	☐	☐
4. copy (or, where required, translation) of statement under Article 19	:	sheets	☐	☐
5. letter	:	sheets	☐	☐
6. other *(specify)*	:	sheets	☐	☐

The demand is also accompanied by the item(s) marked below:

1. [x] fee calculation sheet
2. [ ] separate signed power of attorney
3. [ ] copy of general power of attorney; reference number, if any:
4. [ ] statement explaining lack of signature
5. [ ] nucleotide and or amino acid sequence listing in computer readable form
6. [ ] other *(specify)*:

Box No. VII SIGNATURE OF APPLICANT, AGENT OR COMMON REPRESENTATIVE

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the demand).

SCHLICH; George William
5 APRIL 2000

For International Preliminary Examining Authority use only

1. Date of actual receipt of DEMAND:
2. Adjusted date of receipt of demand due to CORRECTIONS under Rule 60.1(b):
3. [ ] The date of receipt of the demand is AFTER the expiration of 19 months from the priority date and item 4 or 5, below, does not apply. [ ] The applicant has been informed accordingly.
4. [ ] The date of receipt of the demand is WITHIN the period of 19 months from the priority date as extended by virtue of Rule 80.5.
5. [ ] Although the date of receipt of the demand is after the expiration of 19 months from the priority date, the delay in arrival is EXCUSED pursuant to Rule 82.

For International Bureau use only

Demand received from IPEA on:

See Notes to the demand form

CHAPTER II

PCT

FEE CALCULATION SHEET

Annex to the Demand for international preliminary examination

International application No.	PCT/GB99/03031
Applicant's or agent's file reference	GWS/20650 E.6574

For International Preliminary Examining Authority use only

Date stamp of the IPEA

Applicant

UNIVERSITY OF EDINBURGH, et al

Calculation of prescribed fees

1. Preliminary examination fee	DEM 1,533.00	P
2. Handling fee *(Applicants from certain States are entitled to a reduction of 75% of the handling fee. Where the applicant is (or all applicants are) so entitled, the amount to be entered at H is 25% of the handling fee.)*	DEM 147.00	H
3. Total of prescribed fees Add the amounts entered at P and H and enter total in the TOTAL box	DEM 1680.00	TOTAL

Mode of Payment

[X]	authorization to charge deposit account with the IPEA (see below)	[ ]	cash
[ ]	cheque	[ ]	revenue stamps
[ ]	postal money order	[ ]	coupons
[ ]	bank draft	[ ]	other *(specify)*:

Deposit Account Authorization *(this mode of payment may not be available at all IPEAs)*

The IPEA/ _____ [X] is hereby authorized to charge the total fees indicated above to my deposit account.

[ ] *(this check-box may be marked only if the conditions for deposit accounts of the IPEA so permit)* is hereby authorized to charge any deficiency or credit any overpayment in the total fees indicated above to my deposit account.

2805.0049	5 APRIL 2000	
Deposit Account Number	Date *(day/month/year)*	Signature

See Notes to the fee calculation sheet

PATENT COOPERATION TREATY

From the:
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

PCT

To:

SCHLICH, George William et al
MATHYS & SQUIRE
100 Gray's Inn Road
London WC1X 8AL
GRANDE BRETAGNE

WRITTEN OPINION

(PCT Rule 66)

Date of mailing *(day/month/year)*	27.06.2000
Applicant's or agent's file reference: GWS/20650	**REPLY DUE** **within 3 month(s)** from the above date of mailing

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB99/03031	13/09/1999	11/09/1998

International Patent Classification (IPC) or both national classification and IPC

C12N5/00

Applicant

UNIVERSITY OF EDINBURGH et al.

1. This written opinion is the **first** drawn up by this International Preliminary Examining Authority.

2. This opinion contains indications relating to the following items:

I	☒	Basis of the opinion
II	☐	Priority
III	☐	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
IV	☐	Lack of unity of invention
V	☒	Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
VI	☐	Certain document cited
VII	☐	Certain defects in the international application
VIII	☒	Certain observations on the international application

3. The applicant is hereby **invited to reply** to this opinion.

When? See the time limit indicated above. The applicant may, before the expiration of that time limit, request this Authority to grant an extension, see Rule 66.2(d).

How? By submitting a written reply, accompanied, where appropriate, by amendments, according to Rule 66.3. For the form and the language of the amendments, see Rules 66.8 and 66.9.

Also: For an additional opportunity to submit amendments, see Rule 66.4.
For the examiner's obligation to consider amendments and/or arguments, see Rule 66.4 bis.
For an informal communication with the examiner, see Rule 66.6.

If no reply is filed, the international preliminary examination report will be established on the basis of this opinion.

4. The final date by which the international preliminary examination report must be established according to Rule 69.2 is: 11/01/2001.

Name and mailing address of the international preliminary examining authority:
European Patent Office
D-80298 Munich
Tel. +49 89 2399 - 0 Tx: 523656 epmu d
Fax: +49 89 2399 - 4465

Authorized officer / Examiner
Steffen, P

Formalities officer (incl. extension of time limits)
Vullo, C
Telephone No. +49 89 2399 8061

I. Basis of the opinion

1. This opinion has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this opinion as "originally filed".*):

Description, pages:

1-28 as originally filed

Claims, No.:

1-45 as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. This opinion has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	1-3,5-7,9-33,40,41,43,45
Inventive step (IS)	Claims	1-45
Industrial applicability (IA)	Claims	

2. Citations and explanations

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Re Item I

Basis of the opinion

None of the figures 1-8, referred to in the description of the present application on pages 13-17, were filed with the present application. Examination is consequently carried out without the information presented in the respective figures.

Re Item V

Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

Reference is made to the following documents:

D1: H. NIWA ET AL.: 'SELF-RENEWAL OF PLURIPOTENT EMBRYONIC STEM ELLS IS MEDIATED VIA ACTIVATION OF STAT3.' GENES & DEVELOPMENT , vol. 12, no. 13, 1 July 1998 (1998-07-01), pages 2048-2060.

D2: WO 97 30151 A (THE UNIVERSITY OF EDINBURGH) 21 August 1997 (1997-08-21)

D3: SAVATIER ET AL.: 'WIHTDRAWAL OF DIFFERENTIATION INHIBITORY ACTIVITY/LEUKEMIA INHIBITORY FACTOR UP-REGULATES D-TYPE CYCLINS AND CYCLIN-DEPENDENT KINASE INHIBITORS IN MOUSE EMBRYONIC STEM CELLS.' ONCOGENE, vol. 12(2), 1996, pages 309-322 (abstract).

D4: D.T. DUDLEY ET AL.: 'A SYNTHETIC INHIBITOR OF THE MITOGEN-ACTIVATED PROTEIN KINASE CASCADE.' PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 92, August 1995 (1995-08), pages 7686-7689.

D5: FUKADA, T.H. ET AL.: 'TWO SIGNALS ARE NECESSARY FOR CELL PROLIFERATION INDUCED BY A CYTOKINE RECEPTOR GP130: INVOLVEMENT OF STAT3 IN ANTI-APOPTOSIS, IMMUNITY, vol.5(5), 1996, pages 449-460.

D6: WO-A-94/24274

D7: T. BURDON ET AL.: 'SUPPRESSION OF SHP-2 AND ERK SIGNALLING PROMOTES SELF-RENEWAL OF MOUSE EMBRYONIC STEM CELLS.' DEVELOPMENTAL BIOLOGY, vol. 210, 1 June 1999 (1999-06-01), pages 30-43.

D8: T. BURDON ET AL.: 'SIGNALLING MECHANISMS REGULATING SELF-RENEWAL AND DIFFERENTIATION OF PLURIPOTENT EMBRYONIC STEM CELLS.' CELLS TISSUES ORGANS, vol. 165, no. 3-4, 1999, pages 131-143.

D3, D5 and D6 were not cited in the international preliminary search report. A copy of the document is appended to the present communication.

Examination has been carried out, assuming a validly claimed priority right. Should the priority of the present application found not to be valid, will the documents D7 and D8 have to be considered relevant to the questions of novelty and inventive step for claims 1-45.

The present application refers to methods of culturing embryonic stem cells (ES) cells, in the presence of one or more compounds which either selectively promote self-renewal (propagation or surviving) of the ES cells and/or inhibit propagation or survival of cells other than ES cells, or both. In specific embodiments, propagation inhibition of said compounds is via a signalling pathway essential for the propagation of non-ES cells but not essential for the propagation of ES cells, involving inhibition of either cyclin dependent entry into S-phase of non-ES cells, SHP-2 enzyme, ras/MAPK cascade or MEK. Furthermore are claimed compositions, uses, culture media, methods for deriving (comprising developing an embryo, either *in vitro* or *in vivo*) or culturing ES cells all referring to the said compounds.

The attention is drawn here to the fact that claims 34-39 refer to methods for deriving ES cells (comprising developing an embryo, either *in vivo* or *in vitro*) that, based on the description of the present application (page 9, first paragraph), can be extended to human stem cells and in consequence also to human embryos. The examiner is currently not aware of any PCT contracting state that would consider such subject-matter as patentable. Examination with regard to novelty, inventive step and industrial applicability is carried out however, disregarding the above, on all claims filed.

Claims 1, 2, 3, 5, 6, 7, 9-11, 12-17, 18-21, 22, 23, 24, 25-27, 28-33, 40, 41, 43, 45 lack novelty under the provisions of article 33(2) PCT for the following reasons.

The method of claim 1 is anticipated by D1 (for a "and" combination of compound, D1, page 2056, left column, last paragraph, culture of ES cells in the presence of both LIF and the MEK inhibitor PD098059), D2 (for a "or" combination of compound, D2, page 2, last paragraph, culture of ES cells in the presence of LIF; page 8-10, bridging paragraph; examples 1.8 and 1.9, pages 20-22) and D3 (for a "and" combination of compound, D3, abstract, culture of DIA/LIF-stimulated ES cells by overexpressing p16Ink4a, a specific

inhibitor of CDK4 and CDK6). For the same reasons, the subject-matter of claim 2 is anticipated by D1 and D3 and the subject-matter of claims 3, 5 and 6, the latter two ones depending on claim 3 and ultimately on claim 1, is anticipated by D1 (same citations, it is noted here that PD098059 is inherently a specific inhibitor of MEK and hence of reduces the activity of the ras/MAPK cascade and can therefore also be considered to reduce the activity of a mitogen activated protein kinase e.g. D4, page 7687, right column and page 7689, left column, paragraph 3, first sentence). Similarly, it can at present not be excluded that the subject-matter of claims 9-11 is anticpated by D1 (same citation). Novelty of claims 12, 14-17 is anticipated by D1 and D3 (same citations) and the subject-matter of claim 13 can at present not be excluded to be anticipated by D1. Novelty of claims 18-21 is for similar reasons also anticpated by D1 (same citation), because for ES culture, a composition according to these claims, must inherently have been used. Novelty of claim 22 is anticipated by the teachings of D1 and D3 (same citations, e.g. PD098059 and p16Ink4a and it can be assumed, that the use of the compounds, due to their biological effects, lead to the obtention of substantially pure cultures of ES cells). Novelty of claims 23-27 is anticipated by D1 (same citation, because here a culture medium containing PD098059 must inherently have been used for the ES cell culture). Similarly, because in the ES cell culture disclosed in D1 (same citation), a culture medium containing PD098059 and LIF was inherently used, claims 28-33 are also anticipated by D1 (same citation). Novelty of claim 40 is anticipated by D1-D3 (see above citations) and claim 41 is anticpated by D1 and D3 (same citations). Claim 43 is anticpated by D3 (same citation) and claim 45 is anticpated by D1-D3 (same citations, it also noted here that in the culture methods of D1-D3, inherently substantially pure cultures of ES cells were obtained).

In summary, claims 1, 2, 3, 5, 6, 7, 9-11, 12-17, 18-21, 22, 23, 24, 25-27, 28-33, 40, 41, 43, 45 lack novelty under the provisions of article 33(2) PCT and consequently lack also inventive activity under article 33(3) PCT.

Moreover in a more general manner, claims 1-45 lack inventive activity under the provisions of article 33(3) PCT for the following reasons.

The present application refers to methods of culturing embryonic stem cells (ES) cells, in the presence of one or more compounds which either selectively promote self-renewal (propagation or surviving) of the ES cells and/or inhibit propagation or survival of cells other than ES cells, or both. In specific embodiments, propagation inhibition of said

compounds is via a signalling pathway essential for the propagation of cells other than ES cells but not essential for the propagation of ES cells, involving inhibition of either cyclin dependent entry into S-phase of non-ES cells, SHP-2 enzyme, ras/MAPK cascade or MEK.

D1 discloses that in ES cells, LIF stimulates gp130 which in turn mediates signals sufficient for ES cell renewal (D1, page 2048, right column). D1 furthermore discloses, that in the context of gp130, by inhibition of MEK, that the MAPK/ERK signalling pathway activation is not involved in the stem cell colony formation in responses to LIF, neither is SHP-2 in the context of the Ras-ERK cascade involved in self-renewal of ES cells (D1, page 2056, left column, last paragraph). D3 discloses that inhibition of CDK4 by p16Ink4a e.g. inhibition of cyclin-dependent entry into S-phase does not arrest growth of LIF-stimulated ES cells (D3, abstract). It is also known in the art, that for cells other than ES cells, the ERK cascade and in the context of gp130, SHP-2 and the activation of MAP kinase are mediators of cell propagation e.g. see for example D4 and D5. In summary the prior art taken together discloses that propagation of non-ES cells is linked in the gp130 context to SHP-2, MAPK/ERK-MEK, whereas this is not the case in ES cells stimulated with LIF (e.g. a compound that selectively promotes self-renewal of ES cells). Furthermore, a constant aim in the ES cell culture is to produce cell populations of a satisfactorily low degree of heterogeneity and to reduce the presence of non-pluripotent, differentiated cell types from the cultures (see D6, page 2, second paragraph). The instantly proposed solution of this problem, as set forth in claims 1 and 2 and the more specific solutions as referred to in claims 3-7, was obvious to the skilled person when considering the prior art disclosures as mentioned above, which either suggest and employ the claimed methods (claims 1, 3, 5-7) or suggest their obviousness (in the case of SHP-2, claim 4). All further claims, referring to the said methods and compounds of claims 1-7, constitute obvious subject-matter in light of claims 1-7 and are considered therefore also as obvious for the skilled person.

In consequence, claims 1-45 lack inventive activity under the provisions of article 33(3) PCT.

Re Item VII
Certain defects in the international application

Claims 29 and 40-42, when considering their respective dependencies, are not in

accordance with rule 6.4(c) PCT.

Re Item VIII

Certain observations on the international application

The following observations concern article 6 PCT (clarity).

Claims 1 and 2 lack clarity in that characterising technical features which define how the methods have to be carried out are missing e.g. what is the compound that promotes self-renewal and propagation inhibition, what is the cell signalling pathway to be inhibited?

In a similar manner, through the use the undefined term "a compound", claims 9, 10, 12, 14, 18, 19, 22, 23, 25, 28, 30, 34, 37, 38, 40, 41, 43 and 45, lack characterising technical features and are therefore unclear.

Claim 7 refers to "reduces the activity of a Mitogen activated protein kinase". This is ambiguous since it is not clear if reference is made to the activity of a kinase directly or also to downstream effects.

Claim 8, depends on claim 1 and refers to "at least two compounds", whereas in claim 1 reference is made to "a compound". This is inconsistent and leads to unclarity when interpreting the subject-matter of both claims, particularly when considering that in claim 1, it is unclear if "a compound which" refers to either "selectively promotes", "inhibits propagation" or both.

Claim 18 is unclear since it cannot readily be appreciated what is understood with a "synergistic amount".

The term "substantially" in claim 22 is prone to subjective interpretation and therefore unclear.

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Correspondence with the EPO on PCT Chapter II demands

In order to ensure that your PCT Chapter II demand is dealt with as promptly as possible you are requested to use the enclosed self-adhesive labels with any correspondence relating to the demand sent to the Munich Office.

One of these labels should be affixed to a prominent place in the upper part of the letter or form etc. which you are filing.

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

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MATHYS & SQUIRE
100 Gray's Inn Road
London WC1X 8AL
GRANDE BRETAGNE

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing *(day/month/year)*	19.12.2000
Applicant's or agent's file reference GWS/20650	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB99/03031	13/09/1999	11/09/1998

Applicant
UNIVERSITY OF EDINBURGH et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

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Form PCT/IPEA/416 (July 1992)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
GWS/20650		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB99/03031	13/09/1999	11/09/1998
International Patent Classification (IPC) or national classification and IPC C12N5/00		
Applicant UNIVERSITY OF EDINBURGH et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 8 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 4 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
07/04/2000	19.12.2000
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Steffen, P Telephone No. +49 89 2399 7307

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB99/03031

I. Basis of the report

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).)*:
Description, pages:

1-28 as originally filed

Claims, No.:

1-29 with telefax of 26/10/2000

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:
see separate sheet

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	14-19
	No:	Claims	1-13, 20-29
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-29
Industrial applicability (IA)	Yes:	Claims	1-29
	No:	Claims	

2. Citations and explanations
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Re Item I

Basis of the opinion

None of the figures 1-8, referred to in the description of the present application on pages 13-17, were filed with the present application. Examination is consequently carried out without the information presented in the respective figures.

The amendments filed with telefax of 26.10.2000 are in accordance with article 34(2)(b) PCT. Examination is based accordingly on the newly filed claims.

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

Reference is made to the following documents:

D1: H. NIWA ET AL.: 'SELF-RENEWAL OF PLURIPOTENT EMBRYONIC STEM ELLS IS MEDIATED VIA ACTIVATION OF STAT3.' GENES & DEVELOPMENT , vol. 12, no. 13, 1 July 1998 (1998-07-01), pages 2048-2060.

D2: WO 97 30151 A (THE UNIVERSITY OF EDINBURGH) 21 August 1997 (1997-08-21)

D3: SAVATIER ET AL.: 'WIHTDRAWAL OF DIFFERENTIATION INHIBITORY ACTIVITY/LEUKEMIA INHIBITORY FACTOR UP-REGULATES D-TYPE CYCLINS AND CYCLIN-DEPENDENT KINASE INHIBITORS IN MOUSE EMBRYONIC STEM CELLS.' ONCOGENE, vol. 12(2), 1996, pages 309-322 (abstract).

D4: D.T. DUDLEY ET AL.: 'A SYNTHETIC INHIBITOR OF THE MITOGEN-ACTIVATED PROTEIN KINASE CASCADE.' PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 92, August 1995 (1995-08), pages 7686-7689.

D5: FUKADA, T.H. ET AL.: 'TWO SIGNALS ARE NECESSARY FOR CELL PROLIFERATION INDUCED BY A CYTOKINE RECEPTOR GP130: INVOLVEMENT OF STAT3 IN ANTI-APOPTOSIS, IMMUNITY, vol.5(5), 1996, pages 449-460.

D6: WO-A-94/24274

D7: T. BURDON ET AL.: 'SUPPRESSION OF SHP-2 AND ERK SIGNALLING PROMOTES SELF-RENEWAL OF MOUSE EMBRYONIC STEM CELLS.' DEVELOPMENTAL BIOLOGY, vol. 210, 1 June 1999 (1999-06-01), pages 30-43.

D8: T. BURDON ET AL.: 'SIGNALLING MECHANISMS REGULATING SELF-RENEWAL

AND DIFFERENTIATION OF PLURIPOTENT EMBRYONIC STEM CELLS.' CELLS TISSUES ORGANS, vol. 165, no. 3-4, 1999, pages 131-143.

D5 and D6 were not cited in the international preliminary search report. A copy of the document has been appended to the written opinion. D3 is cited in the application (page 11, line 20).

Examination has been carried out, assuming a validly claimed priority right. Should the priority of the present application found not to be valid, will the documents D7 and D8 have to be considered relevant to the questions of novelty and inventive step for claims 1-45.

The present application refers to methods of culturing embryonic stem cells (ES) cells, in the presence of one or more compounds which either selectively promote self-renewal (propagation or surviving) of the ES cells and/or inhibit propagation or survival of cells other than ES cells, or both. In specific embodiments, propagation inhibition of said compounds is via a signalling pathway essential for the propagation of non-ES cells but not essential for the propagation of ES cells, involving inhibition of either cyclin dependent entry into S-phase of non-ES cells, SHP-2 enzyme, ras/MAPK cascade or MEK. Furthermore are claimed compositions, uses, culture media, methods for deriving (comprising developing an embryo, either in vitro or in vivo) or culturing ES cells all referring to the said compounds.

Claims 14-19 are not anticipated by the prior art documents on file. The corresponding subject-matter thus meets with the requirements of article 33(2) PCT.

Claims 1-13 and 20-29 lack novelty under the provisions of article 33(2) PCT for the following reasons.

The method of claims 1-6 is anticipated by D1 (D1, page 2056, left column, last paragraph, culture of ES cells in the presence of both LIF and the MEK inhibitor PD098059). Although D1 is silent towards synergistic action of both compounds as depicted in claim 1, it discloses the simultaneous use of both compounds as depicted in dependent claims 4 and 6. It is therefore inevitable to assume that in the method as described in D1, synergistic effect of both compounds LIF and PD098059 is inherent, even if this parameter was not assessed. For claims 2 and 3, it is noted here that PD098059 is inherently a specific

inhibitor of MEK and hence of reduces the activity of the ras/MAPK cascade and can therefore also be considered to reduce the activity of a mitogen activated protein kinase e.g. D4, page 7687, right column and page 7689, left column, paragraph 3, first sentence). Claim 7 is also at present assumed to be disclosed in D1, as with the information given, the skilled person would immediately recognize an acceptable range of concentration for LIF to be used. It cannot also at present not be excluded, that the disclosure of D3 (D3, abstract, culture of DIA/LIF-stimulated ES cells by overexpressing p16Ink4a, a specific inhibitor of CDK4 and CDK6) meets with all the requirements of claims 1-3 and 5-7. For evident reasons, the disclosures of D1 and D3 can also be considered novelty anticipating for claims 8-13 and claims 8, 9 and 11-13, respectively. Novelty of claims 20-24 are for similar reasons also anticipated by D1 (same citation), because for ES culture, a composition according to these claims, must inherently have been used. Referring also to the above, novelty of claims 20-22 and 24 are at present considered to be anticipated by D3 (same citations). Novelty of claim 25 is anticipated by the teachings of D1 and D3 (same citations, e.g. PD098059 and p16Ink4a and it can be assumed, that the use of the compounds, due to their biological effects, lead to the obtention of substantially pure cultures of ES cells from mixtures of differentiated cells and pure ES cells). For similar reasons as set out above, claims 26-29 are considered to be anticipated by D1 and claims 26, 27 and 29 by D3 (same citations, it also noted here that with the use of both compounds of D1 (PD098059 and DIA/LIF) and D3 (p16Ink4a and LIF), inherently substantially pure cultures of ES cells must have been obtained.

In summary, claims 1-13 and 20-29 lack novelty under the provisions of article 33(2) PCT and consequently lack also inventive activity under article 33(3) PCT.

Moreover in a more general manner, claims 1-29 lack inventive activity under the provisions of article 33(3) PCT for the following reasons.

The present application refers to methods of culturing embryonic stem cells (ES) cells, in the presence of one or more compounds which either selectively promote self-renewal (propagation or surviving) of the ES cells and/or inhibit propagation or survival of cells other than ES cells, or both. In specific embodiments, propagation inhibition of said compounds is via a signalling pathway essential for the propagation of cells other than ES cells but not essential for the propagation of ES cells, involving inhibition of either cyclin dependent entry into S-phase of non-ES cells, SHP-2 enzyme, ras/MAPK cascade or MEK.

D1 discloses that in ES cells, LIF stimulates gp130 which in turn mediates signals sufficient for ES cell renewal (D1, page 2048, right column). D1 furthermore discloses, that in the context of gp130, by inhibition of MEK, that the MAPK/ERK signalling pathway activation is not involved in the stem cell colony formation in responses to LIF, neither is SHP-2 in the context of the Ras-ERK cascade involved in self-renewal of ES cells (D1, page 2056, left column, last paragraph). D3 discloses that inhibition of CDK4 by p16Ink4a e.g. inhibition of cyclin-dependent entry into S-phase does not arrest growth of LIF-stimulated ES cells (D3, abstract). It is also known in the art, that for cells other than ES cells, the ERK cascade and in the context of gp130, SHP-2 and the activation of MAP kinase are mediators of cell propagation e.g. see for example D4 and D5. In summary the prior art taken together discloses that propagation of non-ES cells is linked in the gp130 context to SHP-2, MAPK/ERK-MEK, whereas this is not the case in ES cells stimulated with LIF (e.g. a compound that selectively promotes self-renewal of ES cells). Furthermore, a constant aim in the ES cell culture is to produce cell populations of a satisfactorily low degree of heterogeneity and to reduce the presence of non-pluripotent, differentiated cell types from the cultures (see D6, page 2, second paragraph). The instantly proposed solution of this problem, as set forth in claims 1-3 and 5 and the more specific solutions as referred to in claims 4, 6 and 7, was obvious to the skilled person when considering the prior art disclosures as mentioned above, which either suggest and employ the claimed methods (claims 1-7) or suggest their obviousness (in the case of SHP-2, claim 3). It is noted here the mere observation of an additional parameter for the method of claim 1 (e.g. "synergistic") cannot be taken in account for inventive step since it is only the result of defined method steps that are carried out, said method steps being obvious over the prior art as mentioned above. All further claims, referring directly or indirectly to the said methods and compounds of claims 1-7, constitute obvious subject- matter in light of claims 1-7 and are considered therefore also as obvious for the skilled person.

In consequence, claims 1-29 lack inventive activity under the provisions of article 33(3) PCT.

Re Item VIII

Certain observations on the international application

The following observations concern article 6 PCT (clarity).

Claims 1, 2 and 8 lack clarity in that characterising technical features which define how the methods have to be carried out are missing e.g. what is the compound that promotes self-renewal and propagation inhibition, what is the cell signalling pathway to be inhibited? Furthermore it is unclear how the parameter "synergistic" is to be construed e.g. when and in which test conditions is the combination of compounds (a) and (b) understood to be synergistic.

Referring to this, claim 26 also lacks characterising technical features in respect to "a compound that enhances the response of ES cells". Here it is also not clear when a "compound" is considered to enhance such "response" and how this is assessed.

Claims 7 and 13 refer to LIF concentrations of "about 5 U/ml". First is the term "about" unclear because subjectively interpretable. Second is a definition for the units U missing, as methods of assessing units can vary from laboratory to laboratory and from supplier to supplier.

In a similar manner, through the use of the undefined term "a compound", claims 1, 2, 8, 14, 15, 17, 18, 20, 21, 25 and 26 (also the term "an agent"), lack characterising technical features and are therefore unclear.

Claim 20 is unclear since it cannot readily be appreciated what is understood with a "synergistic amount".

The term "substantially" in claim 25 is prone to subjective interpretation and therefore unclear.

CLAIMS

1. A method of culture of embryonic stem (ES) cells comprising maintaining a culture of ES cells in the presence of:-

(a) a compound that promotes propagation or survival of ES cells; and

(b) a compound that inhibits propagation or survival of cells other than ES cells;

wherein the combination of compounds (a) and (b) is synergistic.

2. A method according to Claim 1 wherein compound (b) selectively inhibits a signalling pathway essential to propagation or survival of cells other than ES cells.

3. A method according to any of Claims 1 or 2, wherein compound (b) is selected from compounds which inhibit activity of the enzyme SHP-2, inhibit the ras/MAPK cascade, inhibit MEK, inhibit a mitogen activated protein kinase or inhibit cyclin dependent entry into S-phase of non-ES cells.

4. A method according to Claim 3, wherein compound (b) is PD098059.

5. A method according to any previous Claims, wherein compound (a) is a cytokine that activates the cytokine receptor gp130 in ES cells.

6. A method according to Claim 5, wherein the cytokine is Leukaemia Inhibitory Factor (LIF).

7. A method according to Claim 6, wherein the concentration of LIF is about 5 U/ml.

8. A method of selecting ES cells from a mixed culture of cells comprising

exposing the mixed culture of cells to:-

(a) a compound that promotes propagation or survival of ES cells; and

(b) a compound that inhibits propagation or survival of cells other than ES cells;

wherein the combination of compounds (a) and (b) is synergistic.

9. A method according to Claims 8, wherein compound (b) is selected from compounds which inhibit activity of the enzyme SHP-2, inhibit the ras/MAPK cascade, inhibit MEK, inhibit a mitogen activated protein kinase or inhibit cyclin dependent entry into S-phase of non-ES cells.

10. A method according to Claim 9, wherein compound (b) is PD098059.

11. A method according to any of Claims 8 to 10, wherein compound (a) is a cytokine that activates the cytokine receptor gp130 in ES cells.

12. A method according to Claim 11, wherein the cytokine is Leukaemia Inhibitory Factor (LIF).

13. A method according to Claim 12, wherein the concentration of LIF is about 5 U/ml.

14. A method of deriving ES cells comprising isolating cells from a non-human embryo or embryoid body and maintaining a culture of those cells in the presence of a compound that selectively inhibits propagation or survival of cells other than ES cells.

15. A method according to Claim 14 comprising dissociating cells obtained and then further maintaining the dissociated cells in the presence of the or a further

compound that selectively inhibits propagation or survival of cells other than ES cells.

16. A method according to Claims 14 or 15 comprising developing an embryo *in vivo*, harvesting the embryo prior to pro-amniotic cavity formation and isolating cells therefrom.

17. A method of deriving ES cells comprising developing a non-human embryo *in vitro*, isolating cells from the inner cell mass of the embryo and maintaining those cells in the presence of a compound that selectively inhibits propagation or survival of cells other than ES cells.

18. A method according to Claim 17 comprising removing primitive endoderm prior to culture in the presence of the compound.

19. A method according to any of Claims 14 to 18, wherein the compound that selectively inhibits propagation or survival of cells other than ES cells is selected from compounds which inhibit activity of the enzyme SHP-2, inhibit the ras/MAPK cascade, inhibit MEK, inhibit a mitogen activated protein kinase or inhibit cyclin dependent entry into S-phase of non-ES cells.

20. A composition for selective culture of ES cells, comprising (a) a compound that inhibits differentiation of ES cells, and (b) a synergistic amount of a compound that inhibits propagation or survival of cells other than ES cells.

21. A composition according to Claim 20 wherein compound (b) selectively inhibits a signalling pathway essential to propagation or survival of cells other than ES cells.

22. A composition according to Claim 20 or 21, wherein compound (b) is selected from compounds which inhibit activity of the enzyme SHP-2, inhibit the ras/MAPK cascade, inhibit MEK, inhibit a mitogen activated protein kinase or inhibit

cyclin dependent entry into S-phase of non-ES cells.

23. A composition according to Claim 22, wherein compound (b) is PD098059.

24. A composition according to any of Claims 20 to 23 wherein compounds (a) is a cytokine that binds to the cytokine receptor gp130, such as LIF.

25. Use of a compound that selectively inhibits propagation or survival of cells other than ES cells in a method of obtaining a substantially pure culture of ES cells from a mixed cell culture.

26. Use of a compound that enhances the response of ES cells to an agent that promotes propagation or survival of ES cells, in a method for a obtaining a substantially pure culture of ES cells.

27. Use according to claim 26, wherein the compound is selected from compounds which inhibit activity of the enzyme SHP-2, inhibit the ras/MAPK cascade, inhibit MEK, inhibit a mitogen activated protein kinase or inhibit cyclin dependent entry into S-phase of non-ES cells.

28. Use according to Claim 27, wherein the compound is PD098059.

29. Use according to Claims 26 to 28, wherein the agent is LIF.